UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

ESTUDIO FUNCIONAL DE FACTORES DE TRANSCRIPCIÓN DE LA FAMILIA MADS-box EN *DICTYOSTELIUM DISCOIDEUM*

TESIS DOCTORAL

MARÍA GALARDI CASTILLA
MADRID, 2012

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MARÍA GALARDI CASTILLA
LICENCIADA EN BIOQUÍMICA POR LA UNIVERSIDAD AUTÓNOMA DE MADRID

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CERTIFICA:

Que doña **María Galardi Castilla**, Licenciada en Bioquímica por la Universidad Autónoma de Madrid, ha realizado bajo su dirección el trabajo titulado:

Estudio funcional de factores de transcripción de la familia MADs-box en *Dictyostelium discoideum*.

Y consideran que el trabajo reúne las condiciones científicas necesarias para ser presentado como Tesis Doctoral.

Y para que así conste a los efectos oportunos, firma el presente documento en Madrid, a ……7…. De …Junio…. De 2012.

Fdo.: Dr. Leandro Sastre Garzón VºBº. Dr Jose Manuel González Sancho

A todos los que me conocen
y a pesar de ello
me quieren

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La verdad es que expresar mis sentimientos nunca ha sido lo mío así que, seguramente cuando leáis esto veréis que no tiene un orden concreto.

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Las desertoras del IDIPAZ. Olga, eres un sol, aunque "maléfica" y perversa como yo… jijiji. Te tengo un grandísimo aprecio. Inma, tu paz interior nos sosiega a todos, gracias a Dios, porque sino…estaríamos atacados de la cabeza. He aprendido mucho de ti, señora. Espero seguir viendo crecer a los enanos, ahora también puedo enseñarles a patinar!!!.

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María V, María P, Pili, Josepo, Marta, Irene, Carlitos, Gema que comidas hemos tenido no?, lo que nos hemos reído y que bodas…BIEN DE BODAS!!! ;). Marta y Alberto además de

lo pasado en el iib tampoco ha sido flojo el viaje a Oslo ehhh?! y los partiditos de padel del martes, muy ricos!!!. Sé que no estaréis por aquí en mi Tesis pero sé que no es por gusto. Disfrutad de la experiencia y ya os iré a ver pronto.

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RESUMEN

La secuenciación del genoma completo de *Dictyostelium discoideum* permitió la identificación de cuatro genes que codifican proteínas con una región similar al dominio MADS-box, *srfA, srfB, srfC y srfD*. Dos de ellos, *srfA* y *srfB*, codifican proteínas similares a SRF, mientras que los otros dos, *srfC* y *srfD*, son más similares a MEF2. En la presente Tesis se ha estudiado la estructura y la función de los genes *srfB* y *srfC* (denominado en la tesis *mef2A*). En *D. discoideum,* srfB está implicado en el comienzo del proceso de desarrollo pluricelular, la migración de las células durante la agregación, la adhesión celular y la culminación de las estructuras. Por otro lado, mef2A es importante para la diferenciación de las células durante el proceso de desarrollo, tanto de las células que darán lugar a las esporas como de un tipo celular que forma parte del tallo. Las funciones realizadas en *D. discoideum* por estos factores guardan cierta similitud con aquellas desempeñadas por sus factores homólogos en animales, SRF y Mef2, que participan en la proliferación y la diferenciación celular de tejidos como el muscular, y la regulación del desarrollo embrionario. En el transcurso de este trabajo también se caracterizó la estructura del gen que codifica la encima Adenilato ciclasa (*acaA*), demostrando que existen tres regiones reguladoras que dirigen la expresión de este gen en diferentes momentos y en regiones específicas de las estructuras en desarrollo. Está bien establecido que *acaA* desempeña una función clave en el proceso de agregación que inicia el proceso de desarrollo pluricelular. Nosotros demostramos que el gen *acaA* se expresa en otros estadios del desarrollo indicando que la encima codificada pudiera desempeñar también otras funciones durante el desarrollo. Además, el estudio de la actividad del gen *acaA* en la cepa mutante para *srfB* ha permitido establecer una posible función reguladora de srfB sobre la expresión del gen *acaA* en determinadas fases del desarrollo como, por ejemplo, el comienzo de la culminación.

SUMMARY

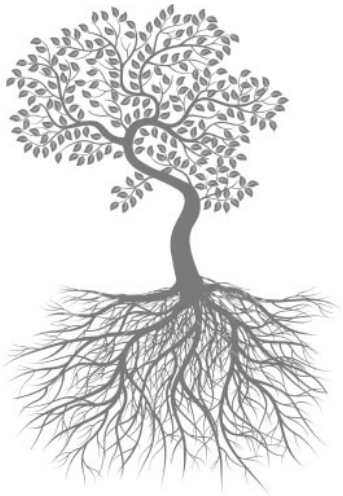
Sequencing of the *Dictyostelium discoideum* genome allowed the identification of four genes coding for proteins with a region similar to MADS-box domains, *srfA, srfB, srfC* and *srfD*. Two of them, *srfA* and *srfB* code for SRF homolog proteins while *srfC* and *srfD* are more similar to MEF*2* proteins. The structural and functional analysis of *srfB* and *srfC* (called *mef2A* from now on) has been approached in this Thesis. In *D. discoideum* SrfB plays an important role at the beginning of developmental process, cellular migration, cellular adhesion and culmination of the structures. On the other hand, Mef2A is important for the cellular differentiation of prespore cells and a group of cells that belong to the stalk structure along development. These functions are similar to those of mammalian SRF and Mef2 proteins that are involved in cellular proliferation and differentiation of muscle tissue and the regulation of embryogenesis. Furthermore, we characterized the structure of *acaA* gene and described the existence of three promoter regions that drive the expression of this gene in different structures and at different times along development. It's well known that acaA plays an important role in the cell aggregation process that initiates *D. discoideum* development. Here we show that acaA is expressed in other developmental stages indicating that it might function in more developmental processes than previously described. The analysis of *acaA* promoter activity in the srfB mutant strain allowed us to identify a possible regulatory function of srfB on acaA gene expression at some of developmental stages such as the beginning of culmination.

Índice

Abreviaturas

ABREVIATURAS

ACA: Adenyl cyclase, Adenilato ciclasa.
ALC: Anterior like cells, células similares a las de la parte anterior.
AMPc: cyclic-Adenosine monophosphate, Adenosina monofosfato cíclica.
CaMK: Calcium/Calmodulin-dependent protein kinase, proteína quinasa dependiente de Calcio y Calmodulina.
cAR: cAMP receptor, receptor de AMPc.
CMF: Conditioned medium factor, factor del medio condicionado.
DBD: DNA binding domain, dominio de unión a DNA.
DIF: Differentiation induction factor, factor inductor de la diferenciación.
GBF: G-box binding factor, factor de unión a caja G.
GPCR: G protein coupled receptor, receptor acoplado a proteína G heterotrimérica.
HAT: Histone Acetyltranferase, Acetiltransferasa de histonas.
HDAC: Histone Deacetylase, Deacetilasa de histonas.
MADS: MCM1, Agamous, Deficiens and SRF.
MAPK: Mitogen-activated protein kinase, proteinas quinasas activadas por mitógenos.
MEF2: Myocyte enhancer factor 2, factor inductor de miocitos 2.
MRTF: Myocardin related transcription factor, factores de transcripción relacionados con miocardina.
NLS: Nuclear localization signal, señal de localización nuclear.
ORF: Open reading frame, pauta de lectura abierta.
PH: Plekstrin homology domain, dominio de homología a pleckstrina.
PI3K: Phosphatidylinositol 3 kinase, fosfatidilinositol-3-quinasa.
PIP3: Phosphatidylinositol-3,4,5-triphosphate, fosfatidilinositol-3,4,5-trifosfato.
PKA: Protein kinase A, proteína quinasa A.
PKD: Protein kinase D, proteína quinasa D.
PLC: Phospholipase C, fosfolipasa C.
PSF: Prestarvation factor, factor previo al ayuno.
PTEN: Phosphatase and tensin homolog, homólogo de fosfatasa y tensina.
SAM: SRF, Agamous and MCM1 domain, dominio de SRF, Agamous y MCM1.
SDF: Spore differentiation factor, factor de diferenciación de esporas.
SRE: Serum response element, elemento de respuesta a suero.
SRF: Serum response factor, factor de respuesta a suero.
SrfA, B, C y D: Serum response factor A, B, C and D of *Dictyostelium discoideum*, Factor de respuesta a suero A, B, C, y D en *Dictyostelium discoideum*.
STAT: Signal transducer and activator of transcription, transductor de señales y activador de la transcripción.
TCF: Ternary complex factor, factor del complejo ternario.
WT: Wild type, tipo silvestre.



Introducción

INTRODUCCIÓN

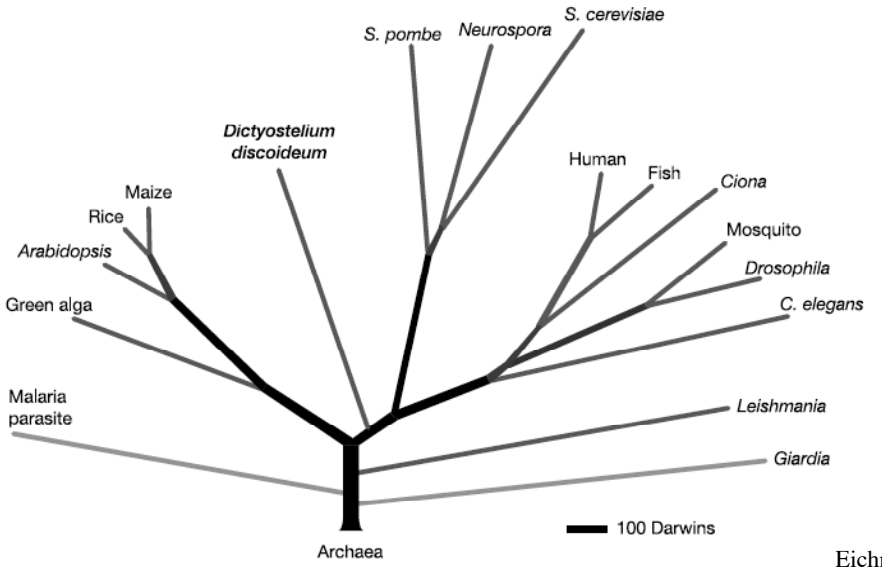
Dictyostelium discoideum

Descubrimiento

Los dictiostélidos son amebas habitantes de suelos forestales donde se alimentan de bacterias y hongos. El primer dictiostélido encontrado fue *Dictyostelium mucoroides* en 1869 por Oskar Brefeld (15) pero *Dictyostelium discoideum* (113) es la especie que, a día de hoy, se sigue utilizando como organismo modelo.

Filogenia

La posición filogenética de *Dictyostelium* (del griego dictyon = red y del latín telium = torre) siempre ha generado una gran controversia. Al principio, se le incluyó dentro del reino de los hongos (7, 74) pero con la llegada de la biología molecular y la secuenciación de genes como *ssu* (18s rRNA), α-tubulina, etc, se llegó a la conclusión de que el género *Dictyostelium* pertenece al grupo filogenético de los *Eumicetozoa*, que diverge del brazo común de animales y hongos después de la separación de las plantas, pero comparte características de los tres grupos **(Fig 1)** (35).



Eichnger et al. 2005

Fig 1. Árbol filogenético de *Dictyostelium discoideum*. Para obtener el árbol filogenético se ha comparado una base de datos de 5279 proteínas de los proteomas de 17 Eucariotas. *Dictyostelium discoideum* pertenece al grupo Eumicetozoa y según este árbol filogenético, *Dictyostelium* se separó del grupo principal formado por plantas, hongos y animales después de la separación de las plantas y antes de la divergencia de animales y hongos.

Características generales.

Dictyostelium discoideum o también llamada la ameba "social", ha sido encontrada en un rango amplísimo de hábitats tan dispares como zonas árticas, regiones tropicales, desiertos y bosques húmedos (114, 127). En esas condiciones vive en forma de ameba solitaria (Fase unicelular) alimentándose por fagocitosis de bacterias y levaduras. Bajo condiciones de ayuno o escasez de alimento sufre un proceso complejo de desarrollo multicelular (Fase multicelular). Las células individuales detectan y secretan un quimioatrayente, la Adenosina monofosfato cíclica (AMPc), para reclutar aproximadamente a 100.000 células a los agregados. Estos agregados o "*mounds"* llevan a cabo un programa morfogenético y de diferenciación celular que concluye con la formación de un cuerpo fructífero o sorocarpo compuesto por un tallo y un esporocarpo que contiene las estructuras de resistencia o esporas. Durante este ciclo tienen lugar procesos similares a los que ocurren durante la embriogénesis de vertebrados, incluyendo migración celular, adhesión, señalización intercelular y diferenciación celular coordinada (37).

Genoma

D. discoideum es un organismo Eucariota, unicelular y haploide en su fase de crecimiento, con un genoma de 34Mb y 6 cromosomas. La secuenciación total de su genoma en 2005 ha permitido estimar en 12.500 los genes que codifica. Se considera uno de los genomas más compactos conocidos; el espacio intergénico medio es de 2.5 kb (128 kb en humanos) y el tamaño de los intrones de 150pb (3500pb en humanos). Es un genoma excepcionalmente rico en nucleótidos de Adenina y de Timidina, en torno a un 78%, porcentaje que disminuye al 60% en las regiones codificantes. Otra característica excepcional del genoma de *D. discoideum* es que presenta gran cantidad de repeticiones del triplete de nucleótidos AAC en las regiones codificantes, lo que da lugar a proteínas con colas de Asparaginas, Glutaminas o Treoninas. El papel que dichas repeticiones juegan, aún se desconoce (35).

Los genes que dan lugar a los ARN ribosómicos 26S, 17S, 5,8S y 5S se encuentran en un elemento extracromosómico de 88kb del que existen 100 copias aproximadamente por núcleo.

El genoma mitocondrial de *D. discoideum* tiene un tamaño de 55kb con un contenido en A-T del 73%. Los genes identificados codifican ARNr, 18 ARNt, 10 subunidades del complejo de la NADH deshidrogenasa, 3 subunidades de la citocromo oxidasa, 4 subunidades del complejo de la ATP sintasa, 15 proteínas ribosomales, apocitocromo b y una serie de ORFs ("*Open Reading Frames"*) de función desconocida. Todos los genes de la mitocondria se transcriben a partir de la misma hebra de ADN. Al igual que en otros organismos, el genoma mitocondrial es muy compacto, estando solapados algunos de los genes (103).

Modelo experimental

D. discoideum ha sido reconocido como modelo experimental por los NIH ("*National Institutes*

of health") debido a la multitud de ventajas que tiene para su uso como tal. Entre dichas ventajas se encuentran, su parecido a las células animales en muchos aspectos estructurales y funcionales, la separación entre fase unicelular y multicelular, lo que nos permite estudiar una gran cantidad de procesos como motilidad, quimiotaxis, agregación o diferenciación. Su ciclo de morfogénesis es sencillo y corto (24h). Sus células se diferencian en dos únicos tipos principales y por último, la aplicación de técnicas moleculares en este organismo es más sencilla debido a su crecimiento en fase haploide. También debemos mencionar que tenemos a nuestra disposición una base de datos para trabajar con *D. discoideum*, http://dictybase.org/.

Ciclo de vida

Dictyostelium discoideum es una ameba social cuyo ciclo de vida consiste en dos fases claramente diferenciadas, crecimiento y desarrollo, la transición entre dichas fases está muy condicionada a la disponibilidad de alimento en el medio.

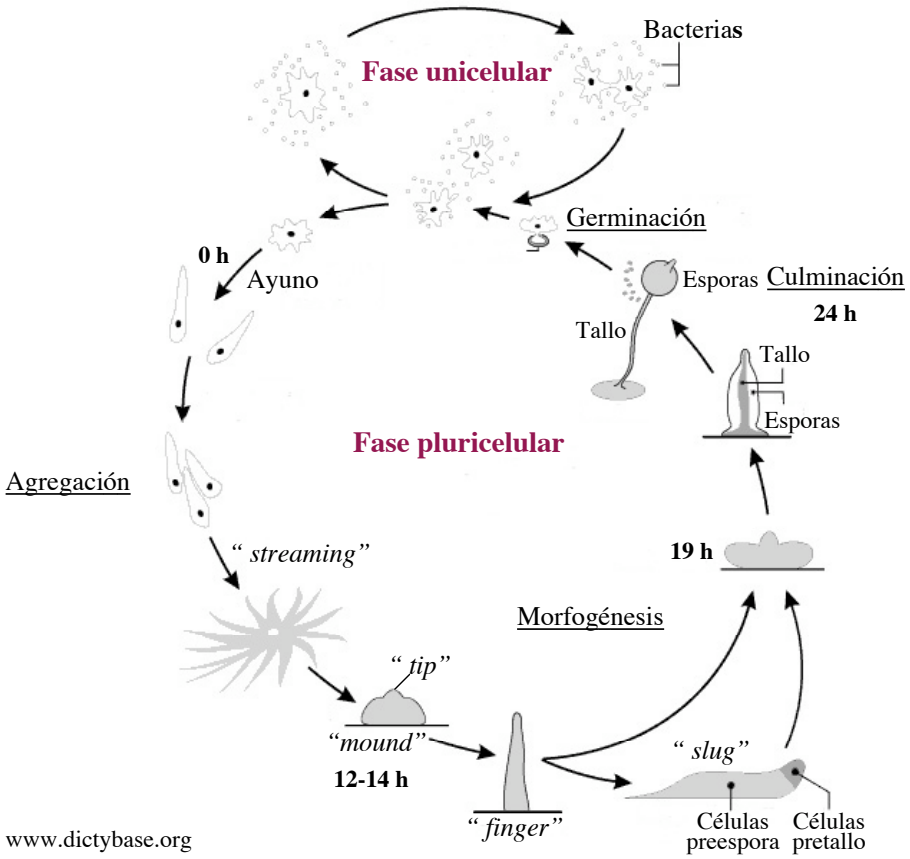


Fig 2. Ciclo de vida de *Dictyostelium discoideum*. Durante la Fase unicelular las células se dividen por fisión binaria cada 8-10 horas y se alimentan de bacterias y levaduras. Cuando escasea el alimento, comienza la Fase pluricelular. Durante la agregación, las células se polarizan y migran ("*streaming"*) hacia los centros de agregación que a su vez son fuente de agentes quimiotácticos como el AMPc, formándose así los agregados o *"mounds"* donde se diferencia el *"tip"* o región organizadora. Durante la morfogénesis, el agregado atraviesa varios estadios. La estructura de migración o *"slug"* es la encargada de buscar las condiciones adecuadas para la culminación. En dicha estructura ya se observa diferenciación celular entre células pre-tallo en la parte anterior y células pre-espora en la parte posterior. La culminación acaba con la formación de un tallo que soporta la caperuza de esporas. Cuando las condiciones ambientales son adecuadas se lleva a cabo la germinación.

- Fase Unicelular

Durante la fase unicelular, *Dictyostelium discoideum* permanece en estado ameboide dividiéndose por fisión binaria simple cada 8-10 horas **(Fig 2)**. En un sustrato sólido, como los suelos de los bosques, se alimenta por fagocitosis de levaduras y bacterias (de las especies *Klebsiella aerogenes, Shigella y* alguna otra). El mecanismo de fagocitosis consiste en el reconocimiento de la bacteria por receptores de membrana y englobamiento en una estructura de membrana llamada fagosoma. El fagosoma, posteriormente, se fusiona al lisosoma y se digiere el contenido (20).

En el laboratorio, se han seleccionado cepas de amebas que crecen en medio líquido sintético (cepas axénicas) captando pequeños volúmenes de medio ("*fluid-phase uptake"*) en estructuras tales como pinosomas o macropinosomas. Este es un proceso que nunca ocurre en la naturaleza (20).

Durante la fase unicelular, las amebas se caracterizan por presentar motilidad y quimiotaxis, es decir, son capaces de moverse en respuesta a un gradiente quimiotáctico mediante la reorganización de su citoesqueleto. Se conocen dos agentes quimiotácticos *en Dictyostelium discoideum*, el ácido fólico, secretado por las bacterias y que les sirve para detectar la cantidad de bacterias en el medio y localizarlas (104), y la adenosina monofosfato cíclica (AMPc), que se produce al inicio de la fase de ayuno, y sirve a las células para agregarse y entrar en la fase de desarrollo multicelular (12) (67).

En el transcurso de la fase unicelular las amebas sintetizan y secretan continuamente factores autocrinos que se acumulan proporcionalmente a la densidad celular existente en el medio (79). A determinadas concentraciones, estos factores desencadenan cambios en la expresión génica que llevan a las células a prepararse para entrar en diferenciación. Las células son capaces de detectar los niveles del factor extracelular PSF ("*Pre-Starvation Factors"),* secretado por las células en crecimiento, y estimar la densidad celular relativa a la cantidad de nutrientes (bacterias) y desencadenar, si es necesario, la entrada en fase multicelular (26, 28).

- Fase Pluricelular

Agregación

Cuando aumenta la cantidad de PSFs y disminuyen los nutrientes en el sustrato, las amebas lo detectan y desencadenan un complejo sistema de respuesta que empieza por la parada de la proliferación. PSF desencadena la expresión de las Discoidinas (27), lectinas implicadas en procesos de adhesión celular necesarios para la correcta polarización y motilidad celular. Otro gen regulado por PSF es un receptor de AMPc (cAR), receptor GPCR ("*G-protein coupled receptor"*) que, acoplado a proteína G heterotrimérica, realiza funciones importantes para la agregación, en concreto cAR1.

Se cree que la expresión génica requiere de la actividad de la proteina-quinasa dependiente de AMPc (PKA, *"c-AMP- dependent protein kinase"*) (118) y que a su vez, los niveles de PSF regulan la traducción de su subunidad catalítica (124).

Las células durante el ayuno también secretan unas glicoproteínas, los CMFs ("*Conditioned Medium Factors"*) que estimulan la expresión génica simultáneamente con PSF (26) y son esenciales para el establecimiento de la señalización de AMPc y el comienzo de la agregación (50, 139). CMF se encarga de controlar la densidad celular durante el crecimiento y de que la estructura final del proceso tenga un número de células adecuado, en torno a 10^5, para que el cuerpo fructífero sea estable y la dispersión de las esporas sea efectiva.

Cuando se dan las dos condiciones, escasez de alimentos y suficientes células en el medio, éstas cesan su proliferación y comienzan el desarrollo mediante un complejo cambio en la regulación de la expresión génica (135). Entre los genes que se inducen se encuentran los que codifican la Adenilato ciclasa (ACA, "*Adenylyl cyclase for agregation"*) que sintetiza AMPc, cARs y la fosfodiesterasa extracelular PDE ("*Phosphodiesterase extracellular"*) que hidroliza AMPc.

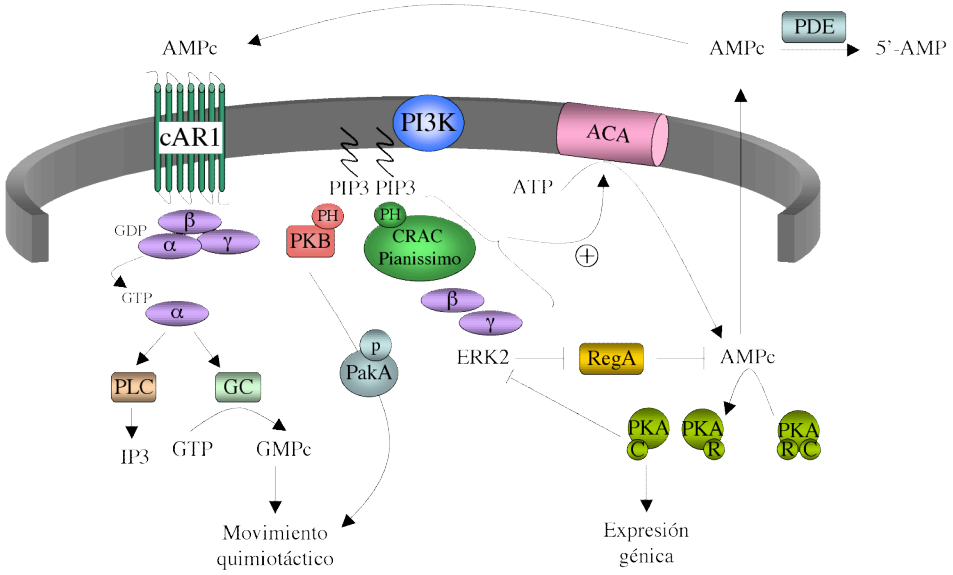


Fig 3. Vías de señalización que regulan la quimiotaxis y la agregación en *D. discoideum*. La molécula de AMPc extracelular al interaccionar con el receptor acoplado a proteínas G, cAR1, provoca el intercambio de GDP por GTP en la subunidad α de la proteína G y la disociación de las subunidades. Esta subunidad α activa a encimas como la Fosfolipasa C (PLC) y la Guanilato ciclasa (GC) implicada en mecanismos relacionados con el movimiento quimiotáctico. La subunidad βγ, a su vez, interacciona con proteínas con dominio PH ("*pleckstrin domain"*) como PKB, CRAC y Pia. PKB fosforila y activa a PakA ("*p21-activated kinase"*) proteína implicada en el ensamblaje de miosina II. CRAC y Pia activan la Adenilato ciclasa (ACA). Esta encima se encarga de sintetizar AMPc. Parte de este AMPc se transloca al medio extracelular donde señaliza a células próximas y sufre degradación a manos de la Fosfodiesterasa extracelular (PDE) silenciando la señal. De hecho, la ACA no se volverá a activar hasta que el AMPc extracelular sea degradado. Por otro lado, el AMPc intracelular controla y activa a la proteína quinasa A (PKA) que regula expresión génica. PKA, a su vez, inhibe a ERK2 que está inhibiendo a la Fosfodiesterasa intracelular (RegA) con lo cual, los niveles de AMPc bajan. Se generan, por tanto, unos ciclos de sensibilización y desensibilización y de pulsos de síntesis de AMPc, que dirigen la agregación hasta la formación del "*mound"*.

Una vez tomada la decisión, unas pocas células comienzan a liberar AMPc en forma de ondas. Las células vecinas detectan el AMPc y responden polarizándose y dirigiéndose hacia la célula que ha emitido la primera onda, formándose unas hileras de células llamadas *"streams"*. A su vez, las células receptoras emiten una nueva onda de AMPc, permitiendo así la amplificación de la señal que llega hasta las células más lejanas. La señal es transitoria, mientras las primeras células se hacen refractarias al AMPc (durante 5-6 minutos) las nuevas son sensibles a él y transmiten la señal (41). Se necesitan del orden de 20-30 pulsos para formar un agregado (en inglés *"mound")*.

La quimiotaxis hacia el AMPc comienza con la llegada de la molécula y su interacción con los receptores de 7 dominios transmembrana, cAR **(Fig 3)**. En agregación, el mayoritario y con mayor afinidad por AMPc, es cAR1 (126). La interacción del AMPc con cAR1 provoca la disociación de la subunidad α y la subunidad $\beta\gamma$ de la proteína G heterotrimérica. Esta vía desencadena varias respuestas (42) (80): a) La activación de la proteína de membrana Adenilato ciclasa (ACA) importantísima para la transmisión de la señal del AMPc y para su propia síntesis. b) La activación de la Guanilato ciclasa (GC) que produce GMPc, necesario para la quimiotaxis y la reorganización del citoesqueleto de actina y miosina (30) (14). c) Activación de la fosfolipasa C (PLC) (11). d) Activación de la proteína quinasa Akt/PKB que se encarga de fosforilar a proteínas PakA ("*p21 activated kinase"*), encargadas del ensamblaje de la miosina (90) (25).

Una de las vías más importantes es la activación, mediada por la subunidad $\beta\gamma$ de la proteína G heterotrimérica (137), de la proteína ACA, con la colaboración de la proteína CRAC ("*Cytosolic regulator of ACA"*) que presenta dominios PH ("*plesktrin homology domain"*) y la proteína Pianissimo (*Pia*) (57) (23) **(Fig 3)**. Para la generación de los agregados se requiere que la vía de señalización del AMPc se adapte rápidamente, para ello, la ACA no puede volver a ser activada hasta que el AMPc extracelular sea degradado por la fosfodiesterasa PDE ("*phosphodiesterase extracellular"*) (54). Las células vuelven a ser sensibles en pocos minutos y estos ciclos de sensibilización-desensibilización dirigen los pulsos de AMPc cada 5-6 minutos durante varias horas hasta la formación de los agregados.

Una fracción del AMPc intracelular activa la PKA ("*cAMP-dependent protein kinase"*). La regulación de los niveles intracelulares de AMPc se cree que también está implicada en la generación de los pulsos de activación de ACA necesarios para la agregación. La fosfodiesterasa intracelular RegA degrada el AMPc y a su vez está regulada por MAPKs (ERK2) que la fosforila y la inhibe, aumentando la cantidad de AMPc intracelular y por tanto activando a PKA. La subunidad catalítica de PKA controla la expresión génica y otras proteínas implicadas en su propia activación (ERK) presentándose así un bucle de regulación **(Fig 3)**.

La activación de la proteína quinasa Akt/PKB es esencial para la quimiotaxis. La activación de estas quinasas depende del receptor de AMPc cAR1, de la proteína G heterotrimérica acoplada a

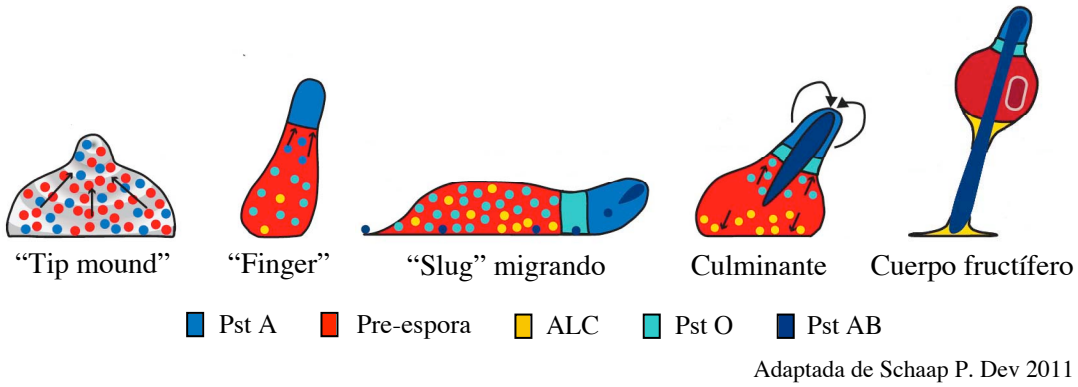
él y de la Fosfatidil inositol-3-quinasa (PI3K). La activación de la PI3K se produce en la parte anterior de la célula, aumentándose así la cantidad de fosfoinositol tri-fosfato o PIP3 que, reclutando proteínas con dominios PH, desencadena una serie de cambios en el citoesqueleto tales como polimerización de actina e inhibición de polimerización de miosina II **(Fig 3)**. En cambio, en la parte posterior de la célula, los niveles de PIP3 se mantienen bajos gracias a la actividad de la fosfatasa PTEN ("*Phosphatase and TENsin homolog"*), que actúa inhibiendo principalmente la polimerización de actina. Este gradiente de PIP3 que se genera en la célula es necesario para que el citoesqueleto se reorganice y las células migren en dirección al gradiente de AMPc (80).

Morfogénesis

Una vez que todas las células han llegado a los centros de agregación, unas 8-10 horas después del comienzo del ayuno, forman una estructura en forma de montaña llamada *"loose mound"* **(Fig 2)** que evolucionará hacia una forma más compacta llamada *"tight mound"* **(Fig 2)**; gracias a mecanismos de adhesión intercelular y a la formación de una capa de polisacáridos y celulosa secretada por las células, y que cubre el agregado. La adhesión está mediada por unas proteínas que son imprescindibles para mantener la estructura multicelular de este organismo y que empiezan a expresarse cuando las células se hacen competentes para agregación (122).

Cuando se ha formado el agregado se ponen en marcha nuevas vías que van a llevar a la diferenciación celular y a la morfogénesis. Se cree que, durante la formación del agregado, la concentración de AMPc extracelular sube hasta llegar a concentraciones constantes del orden de micromolar. En esas condiciones los receptores estarían saturados. A pesar de ello, hay dos factores de transcripción activados por las altas concentraciones de AMPc a través de cAR1, el factor de unión a caja G (GBF, "*G-box binding factor"*) y el factor transmisor de la señal y activador de la transcripción DdSTATa ("*D. discoideum signal transducer and activator of transcription"*). GBF se une a una secuencia de 8 bases que se encuentra en la mayoría de genes inducidos después de la agregación, incluyendo genes específicos de células pre-tallo y de células pre-espora, y genes implicados en uniones intercelulares como *lagC* (16, 49, 56, 116). Durante estas fases tempranas del desarrollo multicelular ya se están empezando a diferenciar los dos tipos celulares que darán lugar a las células tallo y a las esporas, las células pre-tallo ("*prestalk"*) y las células pre-espora ("*prespore"*). Además las células ya se encuentran distribuidas diferencialmente a lo largo del *"mound"*, las pre-tallo en la zona superior y las pre-espora en la inferior **(Fig 4)**. La parte superior de la estructura, es decir las células pre-tallo, forma un abultamiento llamado *"tip"*. El "*tip"* constituye un importante centro de regulación, es la fuente de muchas señales que controlan la distribución de las células; además de inhibir la aparición de otros *"tips"*. Es más, cuando es eliminado, el desarrollo se para hasta que aparece

otro nuevo. Así, el *"tip"* de *D. discoideum* podría considerarse el equivalente al organizador de Mangold/Spemann de vertebrados.



Adaptada de Schaap P. Dev 2011

Fig 4. Distribución de los distintos tipos celulares a lo largo del desarrollo de *D. discoideum*. En la fase de *"tip mound"* ya se diferencian dos tipos celulares, las células pre-espora (rojo) y las pre-tallo pstA (azul). En la fase de *"finger"* se distinguen las células pre-espora, las pstA, las *"anterior like cells"* o ALC (amarillo) y comienzan a verse las pst O (azul claro). En la fase de *"slug"* migrando además de las ya mencionadas comienzan a observarse las células pretallo pstAB (azul oscuro) que constituyen la población de células del "tip-organizer". En los culminantes se empiezan a apreciar como las células comienzan a moverse para buscar su posición definitiva que es la que se observa en el cuerpo fructífero. El tallo formado por las células diferenciadas a partir de las células pre-tallo (pstAB, pstA y pstO), el soro formado por esporas diferenciadas a partir de células pre-espora y sujetado, a su vez, por el disco basal o pie, el *"upper y lower cup"* que se diferencian a partir de las ALC.

El agregado se va alargando para formar el *"finger"*. Esta estructura tiene dos posibilidades en función de las condiciones ambientales. Si la luz proviene de la parte superior y el pH es óptimo, la estructura continuará su desarrollo formando el cuerpo fructífero a las 24 horas. Sin embargo, cuando el pH aumenta y la iluminación es lateral, el *"finger"* se tiende sobre el sustrato y forma una estructura migratoria (*"slug"*) **(Fig 2 y Fig 4)**.

Migración del slug

El *"slug"* es una estructura capaz de moverse por fototaxis y termotaxis a lo largo del sustrato hasta que las condiciones sean las favorables para culminar y formar el cuerpo fructífero. Son capaces de migrar hacia luz de longitud de onda entre 425 y 550 nm, que corresponden al espectro visible, mientras que dentro del rango ultravioleta se alejan de la fuente de luz (43, 108). En cuanto al movimiento termotáctico, son capaces de migran hacia fuentes de entre 25-30 grados centígrados y distinguen variaciones de temperatura de 0,0009ºC/cm (13, 109). En esta etapa del desarrollo la diferenciación y la localización celular están claramente determinadas **(Fig 4)**. Mientras que en la parte anterior se encuentran las células pre-tallo (20% anterior del *"slug"*), en la parte posterior se encuentran las células pre-espora (80% posterior del *"slug"*). Dentro de la parte anterior o pretallo se diferencian varios subtipos celulares, pstA,

pstAB y pstO. Dichos subtipos se caracterizan por presentar diferentes patrones de expresión de algunos genes específicos de matriz extracelular como *ecmA* y *ecmB*.

En la parte posterior se encuentran las células pre-espora dentro de cuya masa se encuentran un tipo de células pretallo, las ALC ("*Anterior Like Cells")*, células similares a las de la parte anterior (37) **(Fig 4)**.

El que un "slug*"* continúe migrando o decida culminar depende de las condiciones del medio **(Fig 2)** y la culminación se desencadena gracias a la actuación de la encima proteína quinasa dependiente de AMPc (PKA). En *Dictyostelium*, la función del AMPc intracelular está mediada por la PKA, proteína importante a lo largo de todo el desarrollo. La inhibición de PKA produce cepas que no culminan y permanecen en estadio *"slug",* mientras que la sobre-expresión de la subunidad catalítica de PKA genera cepas que se desarrollan más rápido que la cepa silvestre y forman esporas precozmente. Otra de las condiciones que regula la culminación es la cantidad de amonio. El amonio es un subproducto generado durante el catabolismo y que es capaz de inhibir la culminación a través de PKA. El amonio es captado por los receptores histidina quinasa que activan la fosfodiesterasa RegA (fosfodiesterasa de AMPc), disminuyendo la cantidad de AMPc interno y a su vez la actividad de PKA, provocando así que se alargue la fase de migración o *"slug"*. Por lo tanto la actividad de la encima PKA es muy importante para que el desarrollo de *D. discoideum* continúe, supere el estadio *"slug"* y se produzca la diferenciación terminal (37).

Culminación

La culminación es la fase final del desarrollo **(Fig 2)** en la cual se forma el cuerpo fructífero y se produce la diferenciación terminal de las células tallo y espora El cometido final del cuerpo fructífero es soportar las formas de resistencia o esporas y favorecer su dispersión a regiones más adecuadas para la germinación.

Cuando comienza la culminación, las células pretallo, de la parte anterior del "*slug"*, forman un tubo que atraviesa la masa de células pre-espora, para llegar al sustrato, anclar la estructura y formar el tallo. Al producirse el crecimiento del tallo la masa de células pre-espora asciende **(Fig 4)**. La diferenciación terminal a células tallo y a esporas es un proceso que sucede gracias a que los factores secretados por un tipo celular son reconocidos o detectados sólo por el otro tipo celular. Así por ejemplo, las células pre-tallo requieren del factor inductor de diferenciación DIF ("*Differentiation Inducing Factor"*), que es una hexafenona clorada secretada por las células pre-espora (62, 63). Por otro lado, los factores de inducción de esporas (SDF, "*Spore Differentiation Factor"*) son necesarios para el encapsulamiento de las esporas (125). El tallo se forma por la vacuolización y muerte de las células pre-tallo. Mientras que la diferenciación a esporas consiste en su deshidratación y la formación de una capa exterior de proteínas y celulosa. Por otra parte las células ALC *"anterior like cells"* darán lugar a la cubierta superior

del soro o *"upper cup"* a la inferior o *"lower cup"* y al disco basal que fija la estructura al sustrato (37) **(Fig 4)**.

Factores de transcripción de la familia MADS-box

Descubrimiento

En 1984 Greenberg observó que la adición de suero a cultivos celulares quiescentes lograba una rápida estimulación de la transcripción de *c-fos*, gen inmediatamente temprano necesario para que las células salgan de quiescencia y puedan iniciar la mitosis (51). En el promotor de este gen se halló una secuencia, localizada a unas 300 bases por delante del inicio de la transcripción, que era suficiente para provocar una respuesta a suero (52, 112). A esta secuencia, caracterizada por tener un núcleo de 6 Adeninas (A) ó Timidinas (T) flanqueadas por Citosinas (C) y Guaninas (G) ($CC(A/T)_6GG$), se la denominó Elemento de respuesta a suero (SRE *"Serum Response Element"*) o caja CArG ("CArG-box"). Richard Treisman identificó y clonó el factor de transcripción que se unía a esta caja y lo llamo SRF (Factor de respuesta a suero del inglés *"Serum Response Factor"*) (102, 128).

Posteriormente se observó que SRF pertenece a una amplia familia de factores de transcripción caracterizada por la presencia de un dominio de unión a ADN y dimerización denominado **MADS-box** por las iniciales de los primeros factores caracterizados, MCM1 de levaduras, Agamous y Deficiens en plantas y SRF en animales (119).

Estructura

Las proteínas que contienen la caja MADS, entre ellas SRF y MEF2 (Factor inductor de miocitos, del inglés "*Myocyte Enhancer Factor 2"),* constituyen una familia de reguladores transcripcionales implicados en diversos e importantes procesos biológicos. Esta clase de proteínas han sido identificadas en levaduras, insectos (*Drosophila melanogaster*), crustáceos (*Artemia franciscana*), nemátodos, vertebrados y amebas sociales (*Dictyostelium discoideum*).

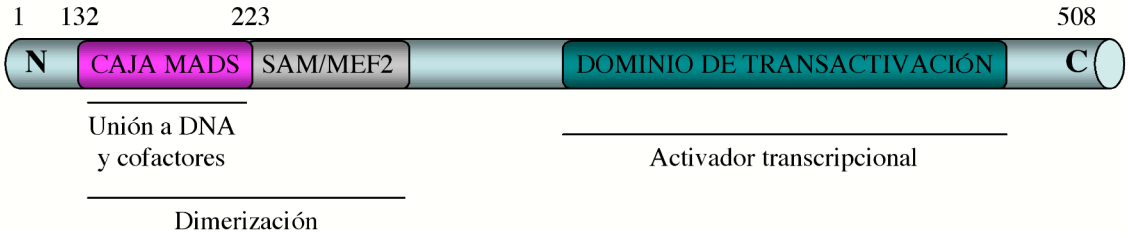


Fig 5. Estructura de los factores de transcripción de la familia MADS. En el extremo amino-terminal entre el aminoácido 132 y el 223, en el caso de SRF humano, se encuentra el dominio MADS encargado de la unión al ADN y la interacción con cofactores transcripcionales. En su proximidad se encuentra el dominio de dimerización, dominio que difiere entre los factores tipo SRF y los tipo MEF2. En el carboxilo-terminal se encuentra el dominio encargado de la activación transcripcional de los genes diana.

Se han identificado dos tipos distintos de proteínas que contienen caja MADS, los similares a SRF (tipo I) y los similares a MEF2 (tipo II). Ambos grupos, tipo I y tipo II, se pueden encontrar tanto en animales como en plantas y hongos. Los dos tipos se caracterizan por la homología que presentan en la región C-terminal inmediata a la caja MADS. Este dominio se llama SAM en proteínas similares a SRF y MEF2 en proteínas similares a MEF2. Las proteínas de un grupo no dimerizan con las de otro grupo (119) **(Fig 5)**.

Los miembros de la familia MADS generalmente reconocen una secuencia rica en Adeninas y Timinas con un núcleo central de 10 pares de bases altamente conservado. Para las proteínas similares a SRF el sitio de unión tiene la secuencia $CC(AT)_6GG$ llamado caja CArG (129); para las proteínas similares a MEF2 el sitio de unión es $CTA(A/T)_4TAG$ (110). La unión de SRF al ADN induce en él una curvatura que favorece el reconocimiento de la secuencia y la conformación correcta de las nucleoproteínas en los promotores y los potenciadores o *"enhancer"* (92).

Las proteínas MADS box son factores de transcripción con capacidad para interaccionar con una variedad muy amplia de cofactores y por tanto son capaces de regular la expresión de múltiples genes y procesos. En muchos casos, el cofactor con el que la proteína MADS box interacciona determina el gen regulado, el momento de la regulación y si son transcripcionalmente reprimidos o activados. La regulación, entonces, es dependiente de su asociación con activadores (92).

Factor de respuesta a suero **SRF** (Serum Response Factor)

- Estructura

En humanos, el gen *srf* tiene un tamaño de 11kb, está localizado en el cromosoma 6 y contiene 7 exones. Por procesamiento alternativo del transcrito primario se obtienen diferentes ARNm que dan lugar a diferentes isoformas. En ratones se han identificado hasta 4 isoformas diferentes dependientes de tejido (64). La isoforma SRF-L contiene los 7 exones y corresponde con la especie de 4.5kb de humanos. La isoforma SRF-M carece del exón 5 y funciona como un mutante dominante negativo que reprime la transcripción dependiente de SRF. La isoforma SRF-S, que carece del exón 4 y del exón 5, sólo se ha identificado en la aorta y la isoforma SRF-I, por último, que conteniendo los exones 1, 2, 6 y 7 sólo se ha localizado en tejidos embrionarios (21).

El factor de transcripción SRF humano es una proteína de 508 aminoácidos y 67 KDa que contiene tres dominios principales: 1. Un dominio de unión a ADN (a la secuencia SRE o "*Serum Response Element*"), 2. Un dominio de transactivación y 3. Varios sitios de fosforilación. En la región Amino-terminal se encuentra el dominio de unión a ADN y

dimerización (*"DNA binding domain"* o dominio DBD), de 90 aminoácidos. Dicho dominio es suficiente para la unión al ADN, la dimerización y la interacción con proteínas cofactores de SRF. Dentro de este dominio, entre los aminoácidos 132 y 197, se encuentra la caja MADS, región altamente conservada evolutivamente y encargada de la unión al surco menor del ADN. Entre *D. melanogaster* y humanos existe una homología del 93% en la caja MADS de SRF. Después de la caja MADS y dentro del dominio DBD, entre los aminoácidos 197 y 223, se encuentra el dominio SAM, dominio funcionalmente implicado en la dimerización del factor de transcripción. El dominio de transactivación está localizado en la región Carboxilo-terminal entre los aminoácidos 339 y 508. Es un dominio de transactivación con una baja capacidad intrínseca pero su interacción con más de 60 cofactores le confiere un potencial de regulación de la transcripción específico de célula y tejido (131, 132). En las proximidades del dominio de transactivación se encuentran unos sitios de fosforilación que son señales de reclutamiento de los cofactores de SRF (97) **(Fig 5)**.

Además de todos estos dominios en el Amino terminal existe una secuencia de localización nuclear (21, 94)

- Funciones de SRF

Proliferación y Diferenciación

SRF está implicado en el control de dos procesos, *a priori* excluyentes, la proliferación y la diferenciación celular. La capacidad, para llevar a cabo el control de estos procesos, reside en la interacción de SRF con una gran diversidad de cofactores, más de 60 descritos.

Experimentos en células en cultivo, usando formas mutantes de SRF, dominantes negativas y RNA de interferencia, para disminuir la expresión de SRF, revelaron importantes funciones para SRF en crecimiento dependiente de suero y en diferenciación de células musculares (123, 134) (60). Un mutante dominante negativo de SRF bloquea la diferenciación de las células del músculo del corazón, en embriones de pollo (69), e impide la diferenciación del músculo cardiaco y esquelético en ratones transgénicos (140).

La mutación en homocigosis de SRF en ratones ($SRF^{-/-}$) es letal embrionaria (5). Los embriones se desarrollan hasta el estadio E6.5, pero a partir de ahí la capa mesodérmica no se desarrolla correctamente dando como resultado la muerte de los embriones entre el estadio E8,5 y E12,5, dado que no progresan más allá de la gastrulación. Las células de los embriones proliferan hasta el día E6.5 y continúan creciendo, incluso en ausencia de SRF, sugiriendo que SRF no es imprescindible para que se produzca la proliferación. SRF regula genes implicados en migración y adhesión (117). Las células madre (ES) o troncales que carecen de SRF presentan defectos en adhesión, migración y en la capacidad de extenderse (del inglés *"spreading"*), todas ellas funciones relacionadas con el funcionamiento del citoesqueleto de actina. Además, presentan disminución en la expresión de genes que son componentes de las fibras de stress

como Talina, Vinculina y diferentes isoformas de Actina; muchos de ellos genes regulados directamente por SRF (117). La temprana letalidad de los ratones KO (del inglés "*Knock out"*) para SRF impide el análisis de las funciones de SRF en el desarrollo del músculo. Se llevaron a cabo, por tanto, experimentos con K.O condicionales en los diferentes linajes musculares. La eliminación condicional de *srf* en el tejido cardiaco causa la muerte a mitad de la gestación, acompañado todo ello de una formación anómala del sarcómero y una regulación incorrecta de genes musculares (96, 101, 106). En el caso del músculo liso, la eliminación de *srf,* lleva a la disminución en la formación de células del músculo liso (del inglés SMC *"smooth muscle cells"*) en la aorta dorsal y las que son detectadas presentan defectos en el citoesqueleto (96). La eliminación de *srf* en el músculo esquelético conlleva la mortalidad en el periodo perinatal provocado por una hipoplasia del músculo esquelético (70). Como puede extraerse de esta información SRF desempeña un papel clave en la formación del citoesqueleto de actina y en la diferenciación de los linajes musculares, esquelético, liso y cardiaco (95).

En *Saccharomyces cerevisiae* existen dos proteínas homólogas a SRF, Mcm1 y Arg80. Arg 80 se necesita para reprimir los genes del anabolismo de la Arginina y activar sin embargo, los genes del catabolismo de la Arginina. (91) Mcm1 también está implicado en el metabolismo de la Arginina pero presenta papeles pleiotrópicos en la célula tales como, el control de la transcripción dependiente de ciclo celular, en la transición entre M/G1 y G2/M (2, 84), el control del apareamiento ("*mating")* (87)*,* el mantenimiento del minicromosoma y otros muchos más.

En *Drosophila melanogaster*, DSRF está implicado en el desarrollo del sistema traqueal que consiste en una estructura ramificada que funciona como sistema respiratorio ya que participa en el intercambio de gases (53). Además, participa en el desarrollo de las alas (99).

SRF en cáncer

Varios estudios muestran una cierta correlación entre el cáncer humano y niveles altos de SRF o de SRF unido a secuencias CArG (24, 105). Si dicho incremento de SRF causa o no el cáncer está por comprobar. Recientemente se ha visto que el microRNA miR-122 se encuentra en menor cantidad en carcinomas hepatocelulares humanos que muestran elevados niveles de SRF (6). Si este miR-122 se vuelve a expresar en células cancerígenas, la tumorogénesis se reduce. miR-122 se une a la región 3' no traducida (3'UTR) de SRF. Cuando se introducía una construcción en la cual SRF carecía de esa región 3' UTR en células que expresaban miR-122 la capacidad inhibitoria de ese micro-ARN se veía reducida, lo cual podría indicar una posible relación entre SRF y el fenotipo cancerígeno (6). En estudios con ARN de interferencia contra SRF y su cofactor MRTF-A (*"Myocardin Related Transcription Factor A"*) se ha observado que se reduce el potencial metastásico de las células mediante el bloqueo de genes implicados en la diseminación, la adhesión y motilidad celular (88). Estos resultados abren una posible vía

de tratamiento para el cáncer usando SRF como diana. De hecho, se ha observado que los elementos CArG son sensibles a la radiación ionizante (29).

SRF y envejecimiento

El papel de SRF en el envejecimiento tiene un carácter pleiotrópico. En el corazón de ratas adultas la cantidad de SRF aumenta tanto en núcleo como en citoplasma con respecto a ratas jóvenes. Los animales jóvenes durante infartos de miocardio reaccionan aumentando la cantidad de SRF, mecanismo que no ocurre igual en adultos. La diferencia en la respuesta puede ser debida a la disminución en los genes tempranos asociada a la edad (77) y por tanto a una menor capacidad de adaptación al *"stress"*. Además se ha observado que en fibroblastos en fase de senescencia la capacidad de unión de SRF al ADN es menor que la de células jóvenes (93). Esto podría correlacionar con la presencia de un SRF hiperfosforilado en células senescentes (18).

- Vías de activación de SRF

SRF presenta varias vías de activación o de regulación, la más común, es la interacción con otros factores de transcripción pero también puede existir fosforilación o regulación de la localización subcelular. Como se menciona anteriormente, SRF regula dos procesos muy importantes y que *a priori* parecen mutuamente excluyentes, la proliferación y la diferenciación celular. La regulación se lleva a cabo gracias a la interacción de SRF con múltiples cofactores que le dotan de especificidad temporal y espacial.

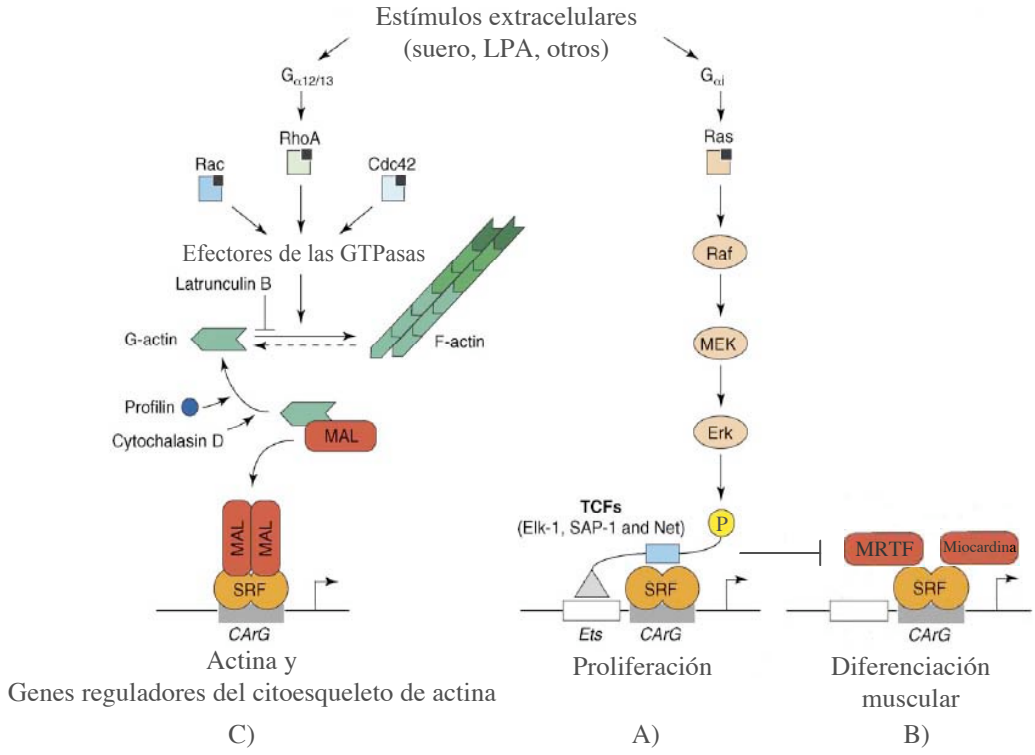
La cristalización de complejos ternarios cofactor-SRF-ADN ha permitido establecer que la unión de SRF y los cofactores produce una curvatura en el ADN, que aumenta considerablemente la unión SRF-ADN. La actividad de SRF también está modulada por unas regiones que flanquean la secuencia de unión CArG. Estas regiones afectan al reconocimiento de la secuencia de ADN, por parte de los complejos SRF-cofactor, y de otros factores accesorios (98).

Las señales extracelulares regulan la actividad transcripcional de SRF a través de dos vías paralelas de señalización. En la primera de ellas, implicada en proliferación **(Fig 6A),** la activación de las MAP quinasas ("*mitogen-activated protein kinase"*) o proteina-quinasas dependientes de mitógeno produce la fosforilación de los TCF o factores del complejo ternario ("*Ternary complex factor"*. Por ej: Elk1, SAP1, etc). Los TCFs pertenecen a la familia de proteínas con dominio Ets. Los TCFs, a través de estos dominios Ets, se unen a unas secuencias de nucleótidos específicas que flanquean las cajas CArG de genes implicados en crecimiento (por ej: *c-fos*). Los TCFs, a su vez, se unen a SRF mediante una región llamada caja B y así potencian la actividad de SRF.

La regulación de la diferenciación está mediada por la interacción con cofactores como GATA-4, TEF-1, Nkx2.5 o Miocardina **(Fig 6B)**. Miocardina y los factores relacionados con

miocardina (MRTF o "*Myocardin Related Transcription Factors"*) pueden incrementar en hasta 1000 veces la capacidad activadora de la transcripción de SRF. Mientras la Miocardina se expresa mayoritariamente en corazón y en células vasculares del músculo liso, los MRTF se expresan en una gran cantidad de tejidos embrionarios y adultos.

Determinados agentes externos activan a las proteínas de la familia Rho de GTPasas (Rho, Rac y Cdc42) que a su vez activan SRF de manera independiente de MAP quinasas y TCF's. Los cofactores de SRF, MRTF-A y -B (MAL o MKL), permanecen secuestrados en el citoplasma mediante su unión a actina G (monómerica). Cuando se activan las GTPasas se induce la polimerización de actina (Actina F), la consecuente liberación de MRTF-A/B y su posterior translocación al núcleo donde se une a SRF y activa la transcripción de genes mayoritariamente específicos de tejidos o de citoesqueleto **(Fig 6C)**.



Adaptada de Posern, G. Trend in Cell Biology 2006

Fig 6. Vías de activación de SRF. **A)** Los factores TCF ("*Ternary complex factor"*) son fosforilados a través de la activación de la vía de las MAP quinasas (ERF, JNK y p38). La fosforilación de los TCF activa la expresión de los genes específicos de proliferación celular a través de SRF, a la vez que impide la unión de factores como la Miocardina o MRTF a SRF (en algunos promotores de genes musculares), **B)** inhibiendo la expresión de genes implicados en la diferenciación muscular. **C)** Los factores MRTF ("*Miocardin related transcription factors"*) también denominados MAL, se encuentran secuestrados en el citoplasma unidos a Actina monomérica (G-actin). La activación de la vía de señalización de las Rho GTPasas (Rac, cdc42 y RhoA) activa la polimerización de actina (F-actin), liberando así los MRTFs que se translocarán al núcleo para unirse a SRF y activar la expresión de genes dependientes de SRF como Actina, vinculina y otros genes del citoesqueleto. De esta manera se genera un bucle de regulación tanto por parte de SRF de genes del citoesqueleto de actina como de ellos sobre la regulación de SRF.

Tanto la Miocardina como los MRTFs tienen una región con una estructura secundaria similar a la caja B de los TCFs y que como tal, compite con ella por su unión a SRF **(Fig 6B)**. Por ejemplo, cuando se estimulan las células del músculo liso con PDGF, Elk1 se fosforila a través de la vía MAPquinasas, facilitándose su asociación con SRF y desplazando a la Miocardina (133), lo que conlleva a una disminución global de la expresión de genes específicos de músculo liso. De acuerdo con esto, bajos niveles de Elk-1 activada, en células de músculo liso, resulta en un incremento de la expresión de genes específicos de músculo (141) (107).

Otro mecanismo de regulación de la actividad de SRF es mediante fosforilación por diferentes quinasas (59) (72). Algunos estudios han demostrado que la unión de SRF al ADN aumenta cuando es fosforilado por la proteína quinasa II (81), sin afectar a la capacidad de dimerización pero si a la conformación de la región de unión al ADN. La mutación de dicho residuo de fosforilación no afecta a la interacción con el sitio CArG pero disminuye la actividad de unión a ADN (82). Otro mecanismo importante para la regulación de la expresión génica es el control de la migración citoplasma-núcleo de los factores de transcripción. La localización de SRF no es estática sino dinámica y modulada por estímulos externos. Por ejemplo, durante la diferenciación de células NIH3T3 en adipocitos (31), SRF es excluido del núcleo. Incluso la regulación de la expresión génica en células de músculo liso se ha visto que está parcialmente controlada por la localización subcelular de SRF (19). Sin embargo, el mecanismo molecular que controla la localización de SRF no se conoce aún. Se ha descrito que la translocación de SRF al núcleo requiere actividad PKA (47) y que la no translocación posiblemente se deba a mecanismos de modificación post-traduccional o incluso de interacción con otras proteínas que de alguna manera enmascaran la señal de localización nuclear (NLS).

Factor inductor de miocitos 2 **MEF-2** (Myocyte Enhancer Factor 2)

- Estructura

Como hemos comentado anteriormente, MEF2 pertenece a la familia de factores de transcripción que poseen caja MADS (119). *Saccharomyces cerevisisae*, *Drosophila melanogaster* y *Caenorhabditis elegans* tienen un único gen *Mef2* , mientras que en vertebrados hay cuatro, *Mef2a, b, c* y *d*. En la región N-terminal de los factores MEF2 se encuentra la caja MADS y en su proximidad el dominio MEF2, ambos juntos están implicados en dimerización, unión al ADN y la interacción con cofactores (10, 86). En la región C-terminal reside la capacidad activadora de la transcripción y es la región donde más divergen los miembros de la familia siendo además objeto de procesamiento alternativo (83) **(Fig 5)**.

Las proteínas MEF2 se unen a una secuencia consenso en el ADN $CTA(A/T)_4TAG$ como homodímeros o heterodímeros (110). Aunque MEF2 es un activador transcripcional depende

también del reclutamiento y la cooperación de otros factores de transcripción para dirigir la expresión de sus genes diana. Además sufre modificaciones post-transcripcionales y post-traduccionales importantes para su función (111).

- Funciones de MEF2

Rlm1, el homólogo de MEF2 en levaduras, se une a la misma secuencia que el factor MEF2 de vertebrados y funciona como un efector de la vía de las MAP quinasas (32), regulando una gran cantidad de genes implicados en la biosíntesis de la pared celular. En *Drosophila melanogaster* el único gen de *mef2* se expresa en mesodermo y posteriormente en diversos linajes musculares, donde está implicado en diferenciación de mioblastos (71). Por otro lado, los cuatro genes de vertebrados presentan patrones de expresión espacial y temporal distintos, pero solapantes, en tejidos embrionarios y adultos, siendo especialmente mayoritarios en músculo estriado y cerebro (34). Además, en vertebrados, MEF2 es también expresado en linfocitos, cresta neural, músculo liso, endotelio y hueso, incluso en algunos estudios lo presentan como ubicuo (111). Los programas de diferenciación celular desencadenados por MEF2 se encuentran en un equilibrio entre las funciones activadoras de la transcripción de él mismo y las funciones represoras que tienen las Histonas deacetilasas de clase IIa (HDACs) (4, 22, 76). En tejidos adultos, las proteínas MEF2 representan un punto clave en la respuesta a stress, programas de remodelación, supervivencia celular, apoptosis y proliferación. En cada una de estas funciones, los genes diana dependen de modificaciones post-transcripcionales específicas de MEF2 y de interacciones con cofactores (111).

- Vías de activación de MEF2

MEF2 está en la confluencia de muchas vías de señalización. Existen dos vías principales de regulación de la activación de MEF2. Las vías de las MAP quinasas convergen en los factores MEF2 en organismos que van desde la levadura al humano (55, 61). La fosforilación del dominio de activación transcripcional de MEF2 por MAP quinasas aumenta su actividad transcripcional, ERK5 (MAPK7) sirve como co-activador de MEF2 por asociación directa (138) **(Fig 7B)**.

Las vías de señalización de calcio también modulan la actividad de MEF2 a través de muchos mecanismos. La actividad de MEF2 está muy controlada por Histonas deacetilasas de clase IIa (HDAC), que en asociación con el dominio MADS, promueve la formación de complejos multiproteicos represores (la cromatina se encuentra condensada) en los genes dependientes de MEF2, como miogenina, metaloproteinasas y mioglobina (86) **(Fig 7A)**. A su vez, diversas calcio-calmodulinas quinasas (CaMKs) y proteína quinasas reguladas por calcio (PKD) fosforilan las histonas deacetilasas de clase II (HDAC). Cuando estas HDAC son fosforiladas se liberan de su unión con MEF2, y se asocian con las proteínas chaperona 14-3-3 que mediante su

unión provocan un cambio conformacional que hace que la señal de localización nuclear (NLS) sea enmascarada y las HDAC exportadas al citoplasma. En ese momento las proteínas Histona acetiltransferasas (HAT) son accesibles a MEF2 y pueden promover la activación de la transcripción de los genes dependientes de MEF2 (la cromatina se encuentra abierta) (85, 86, 111).

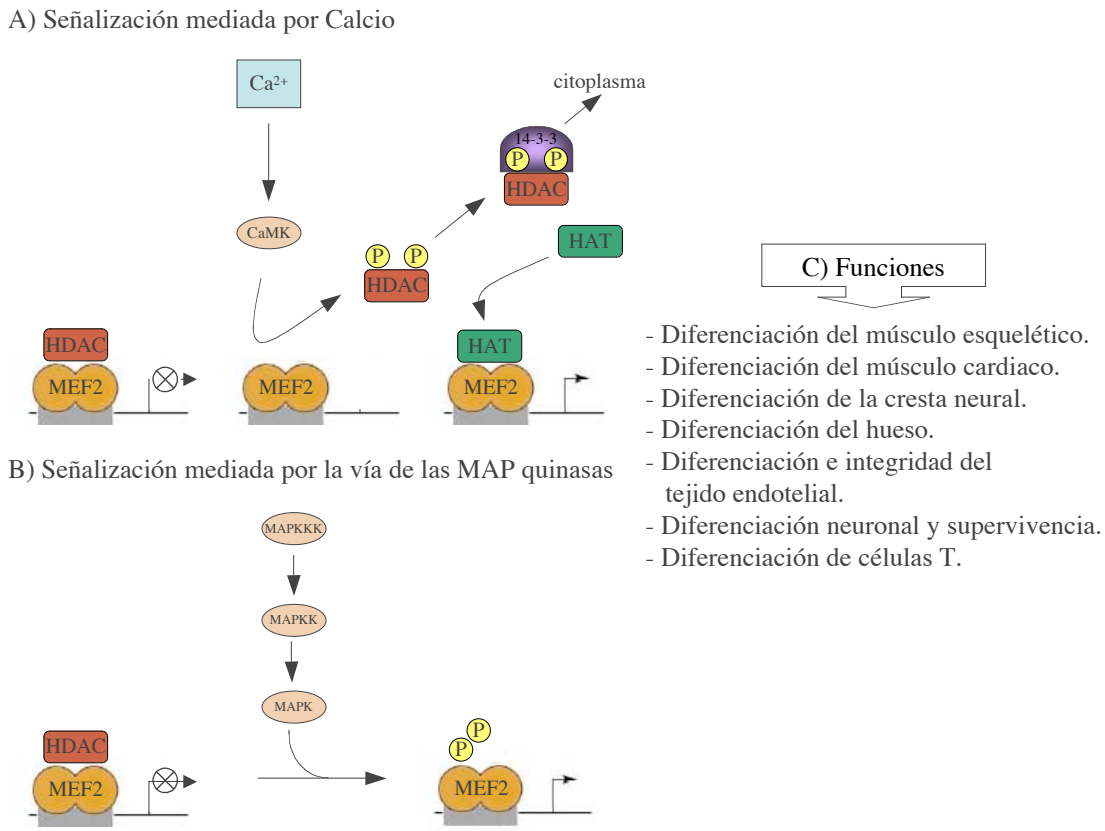


Fig 7. Vías de activación de MEF2 **A)** Las vías de señalización mediadas por calcio activan la transcripción de genes dependientes de MEF2 a través de la Calcio-Calmodulina quinasas (CaMK). La CaMK fosforila las Histonas deacetilasas de tipo II (HDAC) que mantienen bloqueada la transcripción (cromatina condensada), desplazándolas de su unión a MEF2. Las HDAC fosforiladas son sustrato de la proteína chaperona 14-3-3; que al unirse, enmascara la señal de localización nuclear y la HDAC se transloca al citoplasma. A continuación, se puede unir la Histona acetiltransferasa (HAT) que acetila y hace posible la transcripción de los genes dependientes de MEF2. **B)** Las quinasas dependientes de mitógeno o MAP quinasas fosforilan directamente MEF2 y activan la transcripción de los genes dependientes de MEF2. **C)** Funciones en las que está implicado el factor de transcripción MEF2 en vertebrados.

Factores de transcripción de la familia **MADS-box en *Dictyostelium discoideum***

Gracias a la secuenciación total del genoma de *D. discoideum* y a estudios previos realizados en nuestro laboratorio, se han encontrado cuatro genes que codifican proteínas con una región similar a la caja MADS **(Fig 8B)**. Dos de ellos, *srfA y srfB*, codifican proteínas similares a SRF, mientras que los otros dos, *srfC* y *srfD*, son más similares a MEF2 **(Fig 8A)**.

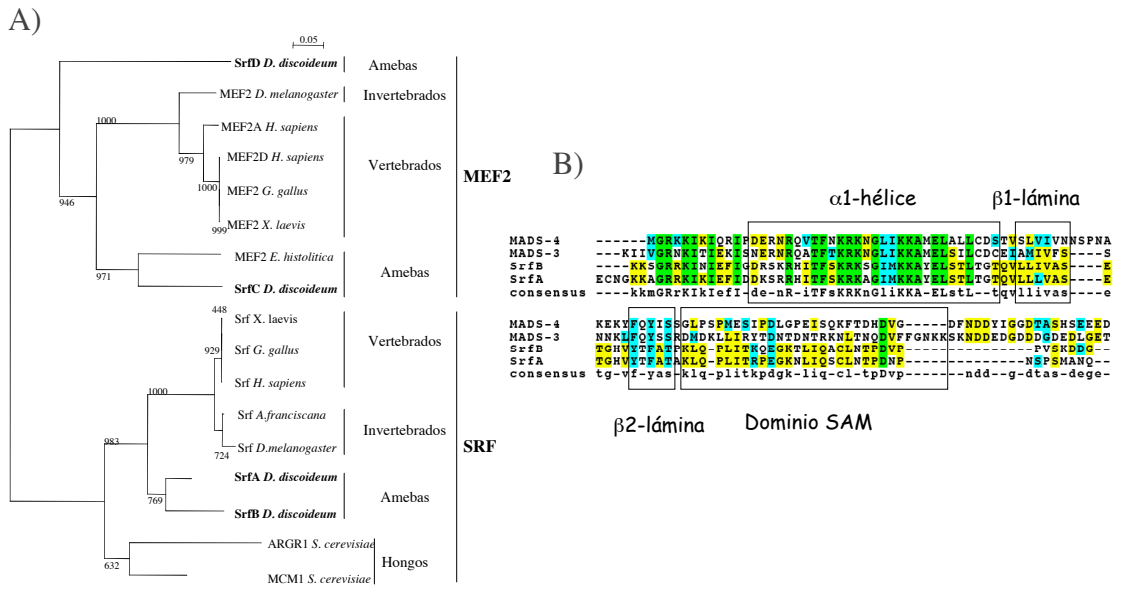


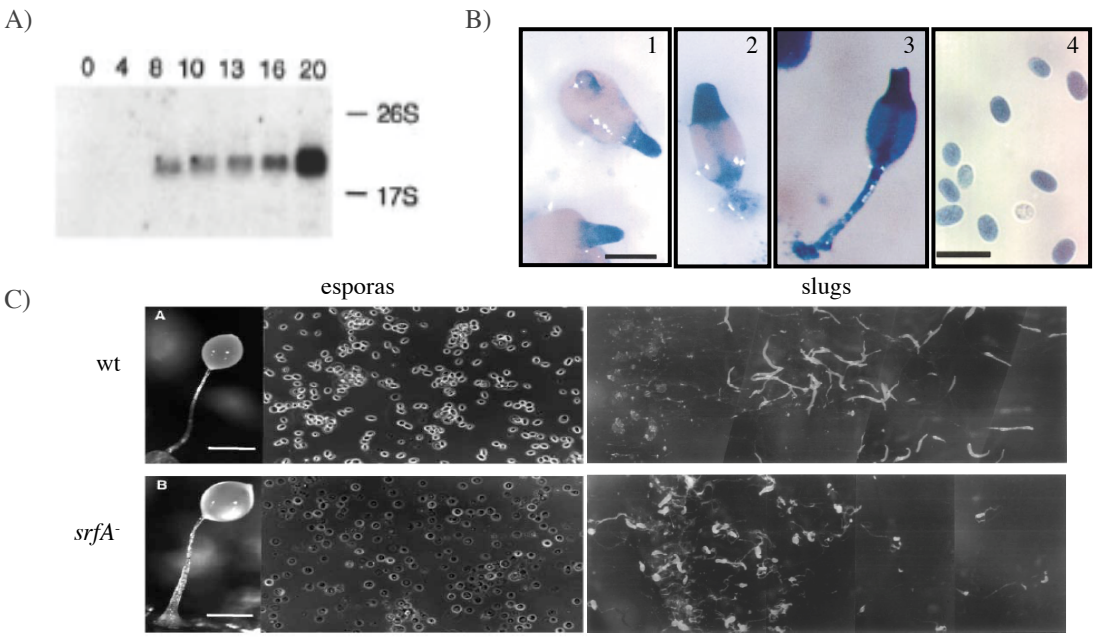
Fig 8. A) Árbol filogenético de los factores de transcripción de la familia MADS box. **B)** Alineamiento de las secuencias de aminoácidos de las proteínas de *D. discoideum* homólogas a SRF (SrfA, SrfB) y a Mef-2 (MADS-3 (SrfC) y MADS-4 (SrfD)).

- *srfA*

El gen *srfA* fue identificado en nuestro laboratorio (36); para ello se usaron unos oligonucleótidos modificados de la región MADS de *D. melanogaster* para amplificar y posteriormente secuenciar un fragmento de 162 pb del ADN de *D. discoideum.* Posteriormente se observó que codificaba una proteína con una gran similitud con el Factor de Respuesta a Suero (SRF) humano en su zona MADS pero también en las regiones próximas.

srfA se expresa durante la segunda mitad del desarrollo de *D. discoideum*. Empieza a partir de las 8 horas cuando las células ya han formado el *"loose mound"* y comienza a existir una cierta divergencia entre los distintos tipos celulares. La expresión se mantiene constante hasta que se produce un pico máximo a las 20 horas que corresponde con el comienzo de la culminación **(Fig 9A)**. También se determinó su expresión a nivel celular y se observó que se expresaba tanto en células pre-tallo como en las células pre-espora y espora hacia el final de la culminación, siendo en estas mayoritaria **(Fig 9B)**. Esto ocurre gracias a la existencia de varias regiones promotoras que regulan su expresión (38).

Para el estudio pormenorizado de sus funciones se construyeron cepas con el gen *srfA* interrumpido y se observó que tenían afectada la diferenciación terminal de las esporas y la migración del slug **(Fig 9C)**. No se forma correctamente la cubierta extracelular de las esporas que las aísla del medio ni tampoco unas fibras de actina que se sitúan en el interior de la célula, lo cual las hace inviables para la supervivencia en condiciones extremas (39). También presentan defectos en la migración de los *"slugs"* o estructuras migratorias, y en la formación del tallo (38).



Escalante, R. Dev 1998 y Escalante, R. Dev. Biology 2001

Fig 9. Caracterización de *srfA* **A)** Expresión del mRNA de *srfA* a lo largo del desarrollo. *srfA* se expresa entre las 8 y las 20 horas del desarrollo de *D. discoideum.* **B)** Análisis de la expresión de *LacZ* bajo el control del promotor entero de *srfA*. Existe expresión en células pre-tallo tanto en *"finger"*(panel1) como en culminates tempranos (panel2). La tinción se empieza a ver en el soro en culminantes intermedios (panel3) y en esporas (panel4). **C)** La cepa mutante *srfA*$^-$ presenta defectos en la formación de las esporas y en su viabilidad (panel izquierdo), además de presentar defectos en la formación y culminación de los *"slugs"* (panel derecho).

El contenido de esta tesis se centró en el estudio y caracterización de otros dos de los genes homólogos de la familia MADS, *srfB* y *srfC*; y el estudio pormenorizado de la región promotora de la encima Adenilato ciclasa (ACA) y su posible regulación por srfB.

Objetivos

OBJETIVOS

1. Caracterización funcional del gen *srfB* de *Dictyostelium discoideum,* homólogo del gen *srf* humano.

2. Descripción detallada de la región promotora del gen de la Adenilato ciclasa (*acaA*), su expresión a lo largo del desarrollo y su control por SrfB.

3. Caracterización funcional del gen *srfC* de *Dictyostelium discoideum*, homólogo del gen *mef2* humano.

Materiales, Métodos y Resultados

MATERIALES, MÉTODOS Y RESULTADOS

Capítulo 1: *SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in Dictyostelium.*

El factor de respuesta a suero (SRF) es un regulador clave durante la proliferación y la diferenciación. Tras la secuenciación del genoma de *D. discoideum* se encontraron 2 genes que codificaban posibles homólogos a SRF. En estudios previos del laboratorio se caracterizó *srfA*. En este trabajo se llevó a cabo la caracterización preliminar del segundo gen homólogo a SRF, *srfB*. El gen *srfB* de *Dictyostelium discoideum* codifica una proteína homóloga a SRF que se expresa en células vegetativas y durante el desarrollo bajo el control de tres promotores alternativos, que muestran patrones de expresión específicos de tipo celular. Los dos promotores más distales dirigen la transcripción génica en la región pretallo PstAB, en el tallo y las regiones por encima y por debajo de la caperuza de esporas (*"upper/lower-cup"*). El promotor más distal es activo durante las primeras horas del desarrollo. Para determinar las funciones de este gen, se construyó una cepa en la cual el gen *srfB* fue interrumpido (*srfB*$^-$). El estudio de la cepa mutante ha revelado que este gen es necesario para la regulación de funciones dependientes del citoesqueleto de actina, como la citocinesis y la macropinocitosis, que no son llevabas a cabo correctamente. Además, la cepa mutante presenta deficiencias en el desarrollo en suspensión, fenotipo que se puede rescatar por adición de pulsos AMPc, lo cual sugeriría un defecto en la señalización por AMPc. La quimiotaxis hacia AMPc de las células *srfB*$^-$ es incorrecta y además, presenta defectos en la inhibición de la formación de pseudópodos laterales. No obstante, las células *srfB*$^-$ agregan en placas de agar y filtros de nitrocelulosa 2 horas antes que las células silvestres y aunque completan correctamente el desarrollo, muestran una mayor tendencia a formar estructuras migratorias que la cepa silvestre. El análisis comparativo de los perfiles de expresión de la cepa silvestre y la cepa mutante mostraron unas diferencias importantes en la regulación de la expresión en condiciones de escasez de alimento (ayuno). De hecho, genes que codifican proteínas ribosomales y lisosomales, genes regulados a lo largo del desarrollo y algunos genes que codifican proteínas implicadas en la regulación del citoesqueleto se encuentran desreguladas en la cepa *srfB*$^-$ durante los primeros estadios del desarrollo de *D. discoideum*. Los datos globales obtenidos en este estudio de caracterización, nos hacen pensar en SrfB como una proteína que ejerce papeles importantes en la regulación del paso de proliferación a diferenciación y en el inicio de la culminación.

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SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in *Dictyostelium*

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Abstract

The Serum Response Factor (SRF) is an important regulator of cell proliferation and differentiation. *Dictyostelium discoideum srfB* gene codes for an SRF homologue and is expressed in vegetative cells and during development under the control of three alternative promoters, which show different cell-type specific patterns of expression. The two more proximal promoters directed gene transcription in prestalk AB, stalk and lower-cup cells. The generation of a strain where the *srfB* gene has been interrupted (*srfB*$^-$) has shown that this gene is required for regulation of actin–cytoskeleton-related functions, such as cytokinesis and macropinocytosis. The mutant failed to develop well in suspension, but could be rescued by cAMP pulsing, suggesting a defect in cAMP signaling. *srfB*$^-$ cells showed impaired chemotaxis to cAMP and defective lateral pseudopodium inhibition. Nevertheless, *srfB*$^-$ cells aggregated on agar plates and nitrocellulose filters 2 h earlier than wild type cells, and completed development, showing an increased tendency to form slug structures. Analysis of wild type and *srfB*$^-$ strains detected significant differences in the regulation of gene expression upon starvation. Genes coding for lysosomal and ribosomal proteins, developmentally-regulated genes, and some genes coding for proteins involved in cytoskeleton regulation were deregulated during the first stages of development.

Keywords: *Dictyostelium*; SRF; Serum Response Factor; Transcription; Cytoskeleton; Actin; Differentiation; Development; cAMP; Aggregation

Introduction

The transcription factor Serum Response Factor (SRF) plays an important role in the regulation of differentiation and proliferation in mammalian cells. It was initially identified because of its role in the regulation of immediate early genes in response to growth factors (Treisman, 1987). Shortly afterwards SRF was found to regulate the expression of muscle-specific genes (Boxer et al., 1989). SRF binds DNA through the highly conserved DNA-binding and dimerization MADS-box domain (MCM1, ArgR1, in yeast, Agamous, Deficiens in plants and SRF in animals) (Treisman, 1995), which is conserved in plants, fungi, animals and amoebae (Alvarez-Buylla et al., 2000; Escalante and Sastre, 1998).

A large number of studies on SRF-dependent gene expression (Zang et al., 2005) and on SRF-binding-site containing promoters have allowed the identification of about 160 genes whose expression is regulated by SRF in mammals (Sun et al., 2007). Besides immediate early genes, SRF-dependent genes include many genes coding for components of the actin cytoskeleton and the muscular contractile apparatus (Miano et al., 2007). SRF regulates the expression of so many different genes through interaction with several signal-regulated and tissue-specific regulatory cofactors (Posern and Treisman, 2006).

Mice in which the SRF gene has been knocked down present embryonic lethality at gastrulation (Arsenian et al., 1998). Cells derived from mutant embryos show defects in cell adhesion, migration and cytoskeleton organization (Schratt et al., 2002).

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Tissue-specific SRF knockdowns have shown that SRF is required for terminal differentiation of skeletal-, cardiac-, and smooth-muscle cells and for the correct organization of the sarcomere (Miano et al., 2004; Li et al., 2005). SRF is also required for migration of neuronal cells (Alberti et al., 2005). These data, in conjunction with other studies in *Drosophila* and *Dictyostelium discoideum* support the proposal of considering SRF as a master regulator of the actin cytoskeleton and contractile apparatus (Miano et al., 2007).

The social amoeba *D. discoideum* belongs to the group of the Eumycetozoa, that diverged from the common branch of animals and fungi shortly after the separation of plants (Eichinger et al., 2005). These organisms grow in forest soils as amoebae but execute a complex multicellular developmental program upon starvation (Maeda, 2005). Cells aggregate by chemotaxis towards cAMP to form little mounds of up to 100,000 cells and undergo a cell-differentiation and morphogenetic program to give rise to a fruiting body consisting of a sorus supported on a cellular stalk. Formation of the fruiting body requires many processes shared with vertebrate embryogenesis, including cell migration, adhesion, inter-cellular signaling and coordinated cell differentiation (Chisholm and Firtel, 2004; Jin and Hereld, 2006).

A *D. discoideum* gene homologous to SRF, *srfA*, was characterized previously (Escalante and Sastre, 1998). The generation of knockdown strains showed that *srfA* is required for several stages of development. The developing structure goes through a stage, the slug, that migrates towards the light. *SrfA* knockdown strains present impaired slug migration (Escalante et al., 2001). These strains also show important defects in spore formation (Escalante and Sastre, 1998; Escalante et al., 2004).

The analysis of the *D. discoideum* genome sequence (Eichinger et al., 2005) allowed the identification of a second SRF homologous gene, *srfB*. In this article the pattern of expression of *srfB* and the structure of the gene are described. The possible biological function of the gene has been approached through the generation of a knockout strain. The strain shows deregulation of actin cytoskeleton functions, such as macropinocytosis and cytokinesis, altered patterns of gene expression at starvation, defects in acquisition of aggregation-specific cell–cell adhesion and chemotaxis, due to impaired cAMP signaling, and deranged post-aggregative development.

Materials and methods

Cell culture, transformation and development

D. discoideum cells were cultured axenically in HL-5 or AX2 medium. Transformation by electroporation was performed as described (Pang et al., 1999). Transformed cells were selected by treatment with blasticidin (Adachi et al., 1994) or neomycin (G418). Filter development was induced by spreading $1–2\times10^7$ cells on filters (Shaulsky and Loomis, 1993). For development on agar plates, a drop of 1×10^6 cells was spread on non-nutrient agar (Pergolizzi et al., 2002). For submerged development $1–2\times10^6$ cells were re-suspended in 2 ml of phosphate-based PDF buffer and placed on plastic multi-well plates (Multiwell, 6 well, FALCON, Becton Dickinson Labware, Franklin Lakes, NJ, USA). For development in shaking suspension, cells were harvested in 0.017 mM Soerensen Na/K phosphate buffer, pH 6.0, resuspended at 1×10^7 cell/ml in Erlenmeyer flasks and shaken at 150 rpm and 23 °C on a rotary shaker (Clim-O-Shaker, A. Kuehner, Birsfelden, Switzerland). Cell treatment with cAMP pulses was done by addition of 2×10^{-8} M cAMP every 6 min, starting within 1 h from beginning of starvation, using a Braun perfusor (Bozzaro et al., 1987).

Generation of knockout, over-expressing and reporter strains

Flanking regions of the *srfB* gene, including nucleotides −954 to 80 and 303–1498, in relation to the start codon were generated by PCR and cloned at both sides of the blasticidin resistance gene in the pGEMT-Easy plasmid vector (Promega). For expression, the coding region of the *srfB* gene, from amino acids 2 to 467, was amplified by PCR and cloned in the pCGFP-CTAP vector (kindly provided by Dr. Pauline Schaap). In a subsequent step the Act15 promoter was substituted by a 2-kb long *srfB* promoter fragment (nucleotides −2152 to 51) generated by PCR.

Putative promoter regions of the *srfB* gene were amplified by PCR and cloned in the PsA-ialphagal vector (Detterbeck et al., 1994), in substitution of the PsA promoter. During amplification of promoter region 1 the last three nucleotides of the 5′UTR, AAG, were changed to ATG to introduce an initiation codon in frame with the reporter gene. β-Galactosidase activity was detected as previously described (Escalante and Sastre, 2006).

Pinocytosis

For each time point, 2.5×10^6 cells were incubated with 2 mg/ml of FITC-dextran (Sigma-Aldrich, St. Louis, MO, USA). Cells were washed, lysed and the incorporated dextran determined in a fluorimeter, as previously described (Rivero and Maniak, 2006). Experiments were made in duplicate and at least three independent experiments were carried out for each strain.

F-actin and nuclear staining

The determination of F-actin was made using the F-actin Visualization kit (Cytoskeleton, Inc., Denver, CO, USA), according to the manufacturer's instructions. After F-actin staining, cells were incubated with 0.1 μg/ml of 4,6-Diamidino-2-phenylindole (DAPI), washed and mounted for microscopic observation (Zeiss Axiophot microscope with Plan-Neofluar objectives).

Northern blot analyses

RNA was isolated using Trizol reagent (Gibco-BRL). RNA transfer to nylon membranes, probe labeling and hybridization were carried out as previously described (Sambrook et al., 1989). DNA probes were generated by PCR using oligonucleotides designed from nucleotide sequences obtained at dicty Base (http://www.dictybase.org).

Rapid amplification of cDNA ends

RNA was isolated from AX4 cells at proliferation or after 8 h of multicellular development. The SMART™ RACE cDNA Amplification kit from Clontech (Clontech Laboratories, Inc., Mountain View, CA, USA) was used for amplification of the 5′ and 3′ untranslated regions of the *srfB* mRNA. The oligonucleotides 5′-CCCTCTTCGCCTGGTGATGATGGAGTTGG-3′, complementary to the region coding for amino acids 20 to 27 of the protein, and 5′-CCACTTCCTTCTATACCATCACC-3′, coding for amino acids 406 to 414, were used. Amplification products were cloned in the pGEMT-Easy vector and the insert from 10 different colonies was sequenced for each of them.

Microarray hybridization

RNA was isolated from AX4 structures after 8 h of multi-cellular development and from *srfB*⁻ structures after 6 h of development. 25 μg of each of three independent RNA samples, from mutant and wild type, were labeled separately with Cy3 and Cy5 by direct incorporation of the dye-dCTP conjugate (GEHealtcare) in a reverse transcription reaction (Superscript III, Invitrogen Carlsbad, CA, USA). The six pairs of complementary labeled cDNAs (three replicates in both dye orientations) were co-hybridized to DNA microarrays comprising 9300 probes printed in duplicate. Arrays were scanned

using an Axon Instruments Genepix 4000B scanner and the resulting images quantified (Genepix 3.0, Axon Instruments) and analyzed using the Bioconductor package Limma (Gentleman et al., 2004; Smyth, 2004, 2005). Background fluorescence was subtracted (Kooperberg et al., 2002), and the resulting log ratios normalized by the print-tip loess function of Limma. The overall log ratio for each gene was obtained using linear model fitting (least squares) and the significance of the apparent differential expression was assessed by a Bayesian approach (Smyth, 2004), adjusting the *p*-values to control the false discovery rate (Benjamini and Hochberg, 1995). In the course of the analysis, a set of genes contiguous on chromosome 2 was found, with a mean log ration shifted to approximately −1, indicating a novel segmental duplication present in the control cells used. These genes were omitted from the list of differentially expressed genes.

Cell adhesion assay

At the end of growth, AX4 and *srfB*-null cells were washed in Soerensen phosphate buffer pH 6.0, resuspended at 1×10^7 per ml and incubated under shaking. At the starvation times indicated cells were washed, resuspended at a final concentration of 1×10^7 per ml and incubated in Soerensen phosphate buffer, with or without 10 mM EDTA, in 0.2 ml volume cuvettes. Cell adhesion was measured by using the light scattering assay and the agglutinometer of Beug and Gerisch, as described (Bozzaro et al., 1987; Bozzaro, 2006).

Chemotaxis assay

Cells starved in shaken culture for 4 to 9 h were plated onto 35-mm diameter glass-based dishes (Iwaki) at a density of approximately 1×10^5 cell/cm^2. Chemotaxis was tested by local stimulation with a microcapillary (Femtotips 1, Eppendorf), filled with a 1.0 mM cAMP solution. Cells were observed with a 20× or a Neofluar 100×/1.3 oil immersion objective, equipped with DIC filter. Images were captured with intervals varying between 0.66 and 1.8 s and recorded in a Panasonic video-recorder (AG-TL7007) with ZVS-47DE camera (Zeiss) mounted on a Zeiss Axiovert HAL200 microscope (Arigoni et al., 2005; Pergolizzi et al., 2002). The recorded time-lapse movies were analyzed using the Image J software. Cell tracks for at least 20 cells in each field (for a total of 170 cells for AX4 and 100 for *srfB*$^-$ cells) were analyzed and cell speed calculated by measuring the distance over time. Orientation was determined by measuring the angle between two subsequent positions of each cell in relation to the position of the cAMP-delivering capillary. Movies showing the migration of AX4 and *srfB*$^-$ cells are included as Supplementary material.

Results

Analysis of the *D. discoideum* genome detected the existence of a second gene coding for a protein similar to MADS-box transcription factors of the SRF family, in addition to the previously characterized *srfA* gene (Escalante and Sastre, 1998). This gene, *srfB*, codes for a protein of 467 amino acids. The predicted amino acid sequence of a region of the protein (amino acids 38 to 118) showed high similarity to the MADS-box and SAM (SRF, Agamous, MCM1) domains of SrfA from *D. discoideum* (86.4% identity), SRF from *Homo sapiens* (72.8%) and *Drosophila melanogaster* (72.8%) and the *Saccharomyces cerevisiae* homologues MCM1 (71.6%) and ARGR1 (56.8%) (Fig. 1A). A phylogenetic tree derived from these sequences

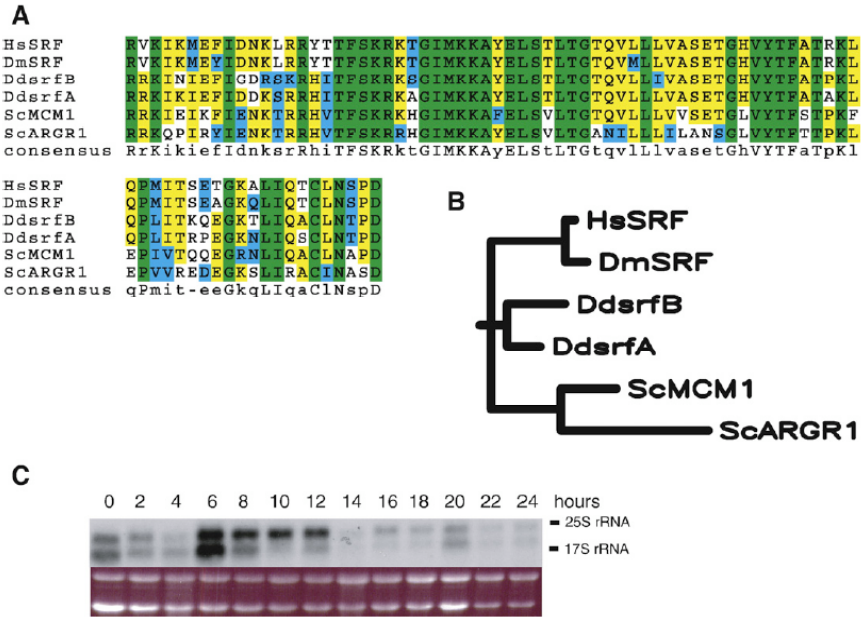
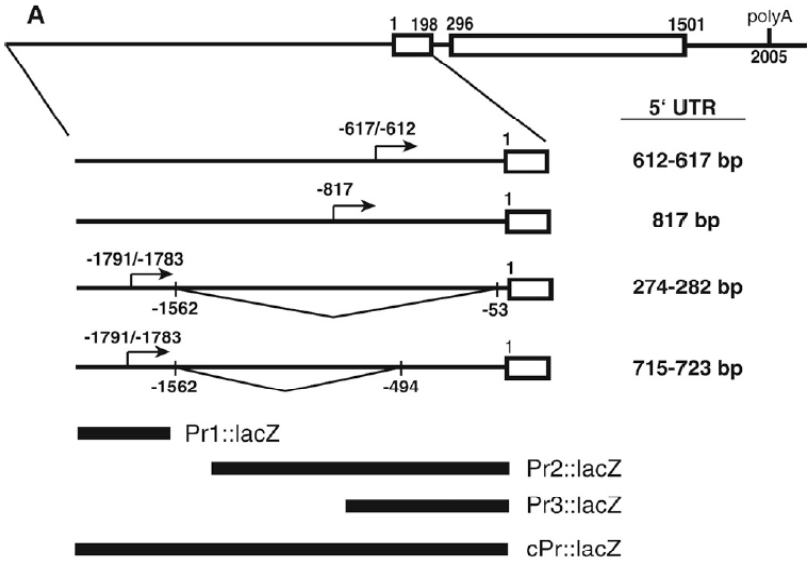


Fig. 1. Identification and expression of the *srfB* gene. Panel A shows the comparison of the deduced amino acid sequences of the *srfB* encoded protein (DdsrfB) with those of *D. discoideum srfA* gene (DdsrfA), human (HsSRF) and *D. melanogaster* (DmSRF) SRF proteins and *S. cerevisiae* Mcm1 (ScMCM1) and ArgR1 (ScARGR1) proteins. Amino acids that are identical in all the proteins are boxed in green and those conserved in 5 of the proteins in yellow. Sequence alignments were made using the ClustalW program at the San Diego Supercomputer Centre (http://www.workbench.sdsc.edu). A phylogenetic tree made from these alignments using the neighbor-joining method, with a generator seed of 111 and 1000 bootstrap trials, is shown in panel B. Panel C, *srfB* expression in proliferating cells (time 0) and at different times of multi-cellular development (2 to 24 h), as determined by Northern blot (upper panel). Migration of ribosomal RNAs is indicated at the right. Ethidium bromide staining of the gel is shown in the lower panel.

also shows that SrfB is more closely related to SrfA than to the animal or yeast genes, indicating a possible duplication of the *srf* gene in Dictyostelids after their divergence from animals and fungi (Fig. 1B).

SrfB expression in proliferating cells and during multicellular development was studied by Northern blot (Fig. 1C). Maximal expression was observed during growth and between 6 and 12 h of development. Two mRNAs were detected that are differentially regulated since the larger mRNA is the predominant species between 8 and 12 h of development.

The origin of the different mRNAs was investigated by the amplification of both cDNA ends. Amplification of the 5′ untranslated region of the cDNA (5′UTR) showed the existence of four different mRNAs coding for the same protein (Fig. 2A). Two of the mRNAs were transcribed from transcription initiation sites located between nucleotides −1783 and −1791 in relation to the ATG initiation codon. Transcripts initiated at these sites were spliced from a donor splicing site located at nucleotide −1562 to two different splice-acceptor sites, located at nucleotides −53 and −494. In addition, two transcription initiation regions, located at −817 and between −612 and −617 were detected. These transcripts were not spliced in their 5′ UTRs. The analysis of the 3′ UTR detected a single polyadenylation site 504 nt downstream of the protein stop codon, that is preceded by a consensus polyadenylation addition signal.



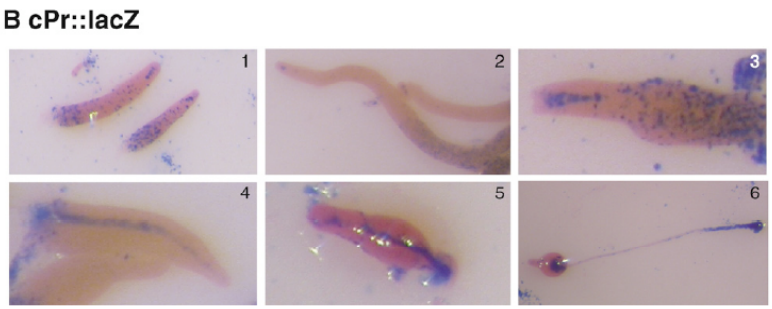


Fig. 2. Structure and cell-type specific function of the *srfB* promoter region. The nucleotide sequence of the *srfB* mRNA 5′ and 3′ untranslated regions was determined by rapid amplification of the cDNA ends (RACE) and the results are schematically shown in panel A. Exon-encoded Open Reading Frames are indicated as boxes and introns and untranslated sequences as lines. The single polyadenylation site identified is indicated (PolyA). The three different transcription initiation regions are indicated by arrows. Two alternative splicing patterns of the mRNAs transcribed from the more distal promoter are indicated. The position of transcription initiation sites, splicing donor and acceptor sites, intron/exon boundaries and polyadenylation site are indicated in relation to the translation initiation codon. The size of the different 5′ untranslated regions of the mRNAs is indicated at the right. The promoter fragments fused to the lacZ reporter gene for expression analyses are schematically indicated (Pr1: :lacZ, Pr2: :lacZ and Pr3: :lacZ). The intergenic region, up to the closest upstream gene, was considered the complete promoter region (cPr: :lacZ). Panel B shows the detection of β-galactosidase activity, indicative of *lacZ* expression, under the control of the complete *srfB* promoter region. The following developmental stages are shown: first finger (1), slug (2), finger (3), early culminant (4, 5) and fruiting body (6).

To determine the spatial and temporal pattern of transcriptional activity, each promoter region (Pr1–3) and the complete promoter region (cPr) were cloned in reporter vectors containing a modified *lacZ* gene coding for unstable β-galactosidase, as schematically shown in Fig. 2A. The intergenic region upstream of the *srfB*-coding region, 4 kb long, was considered as the complete promoter region in these studies. The region of promoter 1 under study contained a 934 nt long fragment upstream of initiation sites −1791/−1783 and also included the mRNA 5′ UTR to the splicing donor site. The Pr2 construct included a 1218 nt region, starting downstream of the splicing donor site up to the initiation codon. The Pr3 construct included a 786 nt fragment from just downstream of the −817 transcription initiation site to the initiation codon.

The complete promoter (cPr) directed *lacZ* expression in vegetative growing cells. Expression decreased during mound formation to be later induced in some cells dispersed throughout the structure (not shown). In later stages of development expression was specifically observed in prestalk cells located at the PstAB region of the first-finger structures, as well as in cells dispersed in the posterior region (Fig. 2B1). Expression was almost completely lost in migratory slugs, except for a few prestalk cells located at the pstAB region (Fig. 2B2). Later on, *lacZ* expression was observed in prestalk cells that were migrating from the top of the structure towards the substrate to form the stalk and in the lower cup region (Fig. 2B3–6). No *lacZ* expression was detected at the prespore region or in the sorus (Fig. 2B5–6).

Promoter 1 directed *lacZ* expression in growing cells, in cells dispersed through the streams (Figs. 3A, B) and in some cells located at the posterior end in early structures (Fig. 3C), but no expression was observed in fruiting bodies (Fig. 3D). In

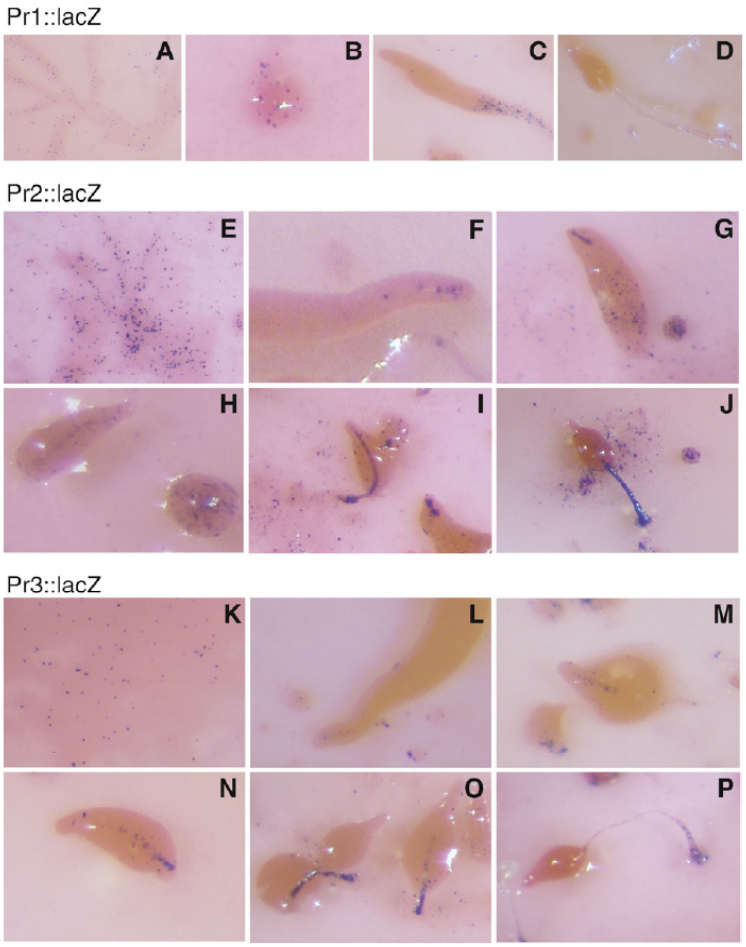


Fig. 3. Developmental regulation of *srfB* promoters. Expression of the *lacZ* reporter gene under the control of *srfB* promoter 1 (A–D), 2 (E–J) or 3 (K–P) was determined by X-gal staining. Aggregating cells (A, E, K), mounds (B), slugs (C, F, L), fingers (G, M), early culminant (H, I, N, O) and culminant structures (D, J, P) were analyzed.

contrast, promoter 2 almost entirely reproduced the pattern of expression of the complete promoter. Therefore, *lacZ* expression directed by promoter 2 was observed in growing cells, in dispersed cells in the streams (Fig. 3E) and in the posterior region of intermediate structures (Fig. 3G), in the PstAB prestalk region of first finger and slug structures (Figs. 3F, G), and in the stalk of early and late culminant structures (Figs. 3H–J). However, the level of *lacZ* expression detected with the Pr2 construct was significantly weaker than that obtained with the complete promoter. Promoter 3 induced a pattern of *lacZ* expression, similar to that of promoter 2, in streams (Fig. 3K), prestalk (Figs. 3L–N) and stalk regions (Figs. 3O, P) but its activity was much weaker.

Generation of a srfB knockout strain

A mutant strain where the *srfB* gene is interrupted was generated to study its function. In this strain, part of the MADS-box coding region of *srfB* (amino acids 27 to 69) was substituted with the blasticidin resistance gene (Fig. 4A). One colony was identified in which the *srfB* gene was interrupted, as shown by PCR analyses (Fig. 4B). Cells from this strain did not express *srfB* mRNAs (Fig. 4C). Mutant cells grew slightly more slowly than wild type (WT) cells on bacteria and in shaken culture with axenic media (data not shown).

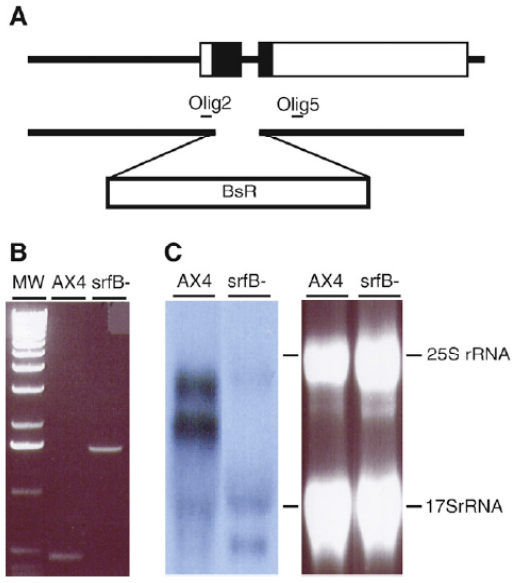


Fig. 4. Generation of a mutant strain where the *srfB* gene has been interrupted. The plasmid construct used for *srfB* interruption and partial deletion is schematically shown in panel A. The *srfB* coding region is indicated as a box in the upper diagram, with the MADS-box coding region shown as a black box. The lower diagram indicates the two genomic regions amplified by PCR and cloned at both sides of the blasticidin-resistance cassette (BsR). The position of the oligonucleotides used for verification of the mutation (Olig2 and Olig5) is indicated. Panel B shows the results of PCR reactions primed with oligonucleotides Olig2 and Olig5 using genomic DNA obtained from wild type (AX4) or *srfB* mutant (*srfB*$^-$) cells. Migration of the 1 kb Molecular Weight Marker (Invitrogen) is indicated (MW). Panel C, the expression of *srfB* mRNA in wild type (AX4) and *srfB* mutant cells (*srfB*$^-$) was analyzed by Northern blot (left panel). The right panel shows the ethidium bromide staining of the gel. Migration of the ribosomal RNAs is indicated at the right.

Mutant cells were able to form cell aggregates on nitrocellulose filters or non-nutrient agar about 2 h earlier than wild type (AX4) cells (Fig. 5A). The expression of some early genes required for aggregation, such as *carA* (coding for the cAMP receptor A) or *acaA* (coding for adenylyl cyclase A) was also induced 2 h earlier in mutant than in WT strains (Fig. 5B).

To determine if the alteration observed was due to the interruption of the *srfB* gene, a plasmid containing the *srfB* gene under the transcriptional control of its own promoter was transfected into the *srfB*$^-$ cells. Two different clones that expressed *srfB* (*srfB*$^-$::*srfB*2 and *srfB*$^-$::*srfB*3) showed a temporal pattern of aggregation on nitrocellulose filters similar to the one of WT cells (Fig. 5A). Mutant cells expressing *srfB* showed a pattern of *carA* and *acaA* expression similar to that of wild type cells, as shown for the srfB$^-$::srfB2 strain (Fig. 5B).

Observation of cell aggregation under submerged conditions detected significant differences between mutant and WT cells. During aggregation WT cells polarized and organized into streams, where the leading edge of one cell contacts the rear end of the preceding one (Fig. 6A). In contrast, *srfB*$^-$ cells extended pseudopodia in different directions during aggregation and hardly formed streams (Fig. 6B). The number of pseudopodia formed by the *srfB*$^-$ strain was determined and compared to that of WT cells. The mutant strain formed significantly more pseudopodia than WT cells (Fig. 6B). Mutant strains expressing *srfB* partially reverted the defect observed, and formed less pseudopodia than the mutant strain (Fig. 6B). When incubated on a glass slide, aggregation-competent WT cells formed very large streams. In contrast, mutant cells formed small clumps from the beginning of starvation, their size increased by accretion and large streams were never formed (data not shown).

The functional significance of the morphological defect observed was further studied by analyzing chemotaxis to cAMP. Starving cells were plated on glass-based dishes, stimulated with cAMP diffusing from a microcapillary and the migration of cells towards cAMP was analyzed. In contrast to WT cells, *srfB*$^-$ cells showed impaired migration after either 7 or 9 h of starvation (movies of the cells are included as Supplementary material). Mutant cells displayed high spontaneous motility in comparison to WT cells but directionality and migration towards cAMP were very deficient (Fig. 7A). *srfB*$^-$ cells stuck strongly to the substrate at the rear end, and moved mainly around their axis, especially after 7 h of starvation. Orientation towards cAMP of the mutant cells improved after 9 h of starvation but is still significantly impaired with respect to WT cells (Fig. 7A).

The finding that *srfB*$^-$ cells, starved in shaken cultures, failed to aggregate normally prompted us to assay their ability to form EDTA-stable contacts. EDTA-stable cell adhesion is developmentally regulated and strictly dependent on the expression of the contact site A glycoprotein on the cell surface (Harloff et

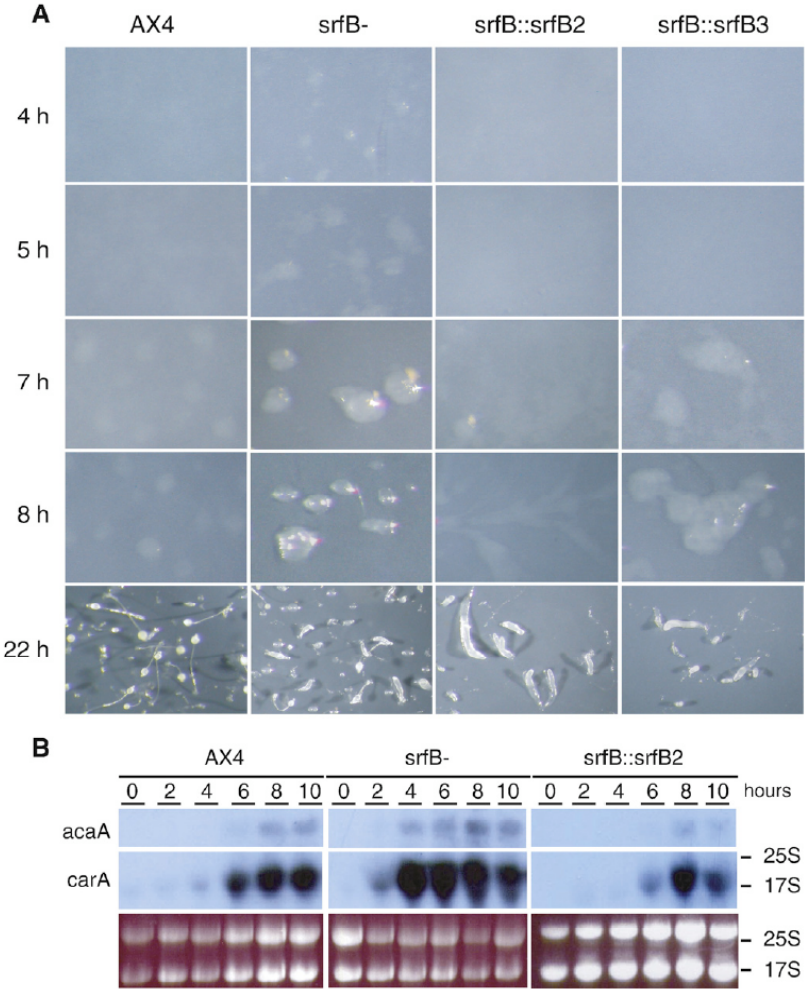


Fig. 5. Early multicellular development of the *srfB* mutant strain. Panel A: Cells from wild type (AX4), *srfB* mutant (*srfB*$^-$) strains and *srfB* mutant strains transfected with a plasmid for *srfB* expression (*srfB*: :*srfB2* and *srfB*: :*srfB3*) were plated on nitrocellulose filters to induce multicellular development. Pictures were taken at 4, 5, 7, 8 and 22 h of development. Panel B: RNAs obtained from wild type (AX4), *srfB* mutant (*srfB*$^-$) and *srfB* mutant strains transfected with a plasmid for *srfB* expression (*srfB*: :*srfB2*) proliferating cells (0) or cells developed on nitrocellulose filters for 2, 4, 6, 8 or 10 h were analyzed for expression of the genes coding for cAMP receptor A (*carA*) or the adenylate cyclase A (*acaA*). Migration of the ribosomal RNAs is indicated for the *carA* blot since the *acaA* mRNA migrated more slowly than both rRNAs. The ethidium bromide staining of the gel is shown in the lower panel.

al., 1989). The encoding *csA* gene belongs to a battery of genes required for aggregation, which are induced at very low level upon starvation and whose expression is strongly stimulated by endogenous cAMP pulses (Gerisch, 1987). Thus, measuring adhesion in the presence or absence of EDTA is a rapid and established method to assess acquisition of aggregation competence (Bozzaro, 2006; Gerisch, 1987).

At the beginning of starvation mutant cells displayed increased EDTA-labile adhesion compared to AX4 cells, which induced rapid formation of small clumps (Fig. 7B). In contrast to the parental strain, which formed EDTA-stable cell–cell contacts by 6 h of development, the mutant cells displayed only a very slight increase in EDTA-stable adhesion even after 10 h of starvation, suggesting strong inhibition in the acquisition of developmentally regulated cell–cell adhesion (Fig. 7B).

To assess whether the defect was due to impaired cAMP signaling, cells were treated with cAMP pulses, supplied every 6 min, thus mimicking the endogenous periodic release of cAMP. As shown in Fig. 7B, cAMP pulsing rescued the mutant, resulting in appearance of EDTA-stable contacts by 6–8 h of development. As expected, acquisition of EDTA-stable adhe-

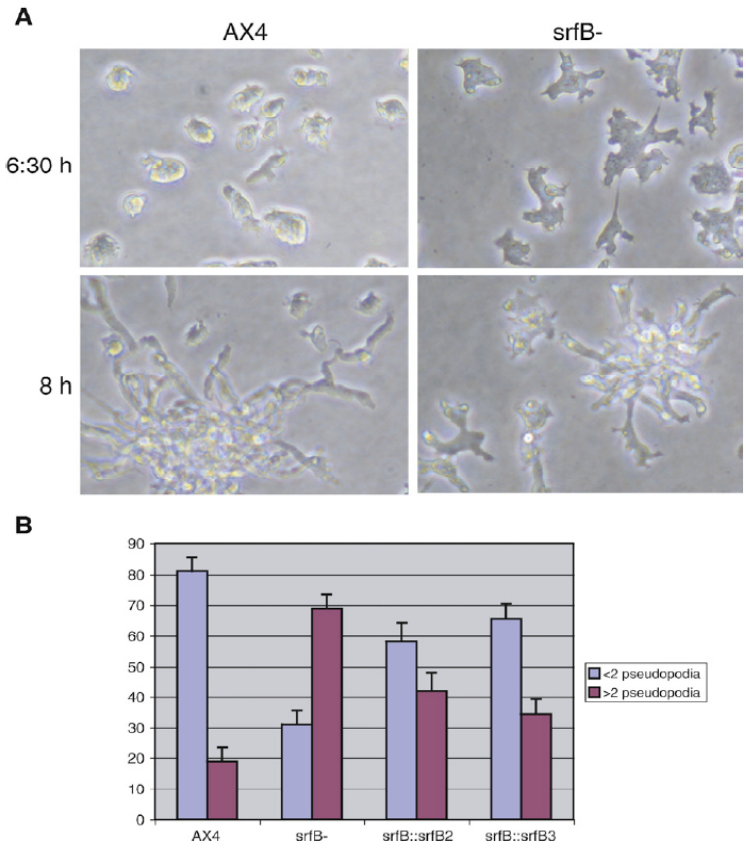


Fig. 6. Aggregation of wild type and *srfB* mutant cells. Panel A: Wild type (AX4) or *srfB* mutant (*srfB*$^-$) cells were incubated on plastic plates for 6:30 or 8 h. Pictures of early stages of cell activation (6:30 h) and cell streaming (8 h) are shown. Panel B: The morphology of wild type (AX4) or *srfB* mutant (*srfB*) cells, and mutated cells that express *srfB* (*srfB*: :*srfB2* and *srfB*: :*srfB3*) was analyzed. Cells that presented 2 or more pseudopodia were quantified in three different experiments. The mean and standard deviation, represented as percentage of analyzed cells, are indicated.

sion was accelerated by cAMP pulses in the parental AX4 cells (Fig. 7B). Thus, these results exclude possible functional defects in contact sites A-mediated cell adhesion or in the ability of the mutant to respond to cAMP pulses. They suggest, instead, that the *srfB*-null cells are impaired in the periodic release of cAMP, which is essential for *csA* gene expression.

In contrast to cell adhesion, the chemotaxis defect was rescued only in part by cAMP pulses. Cells polarized and oriented more rapidly toward the capillary, but the strong cell attachment to the substrate at the rear end persisted for most cells and the rate of chemotactic motility toward the capillary was thus only slightly increased (*data not shown*). More important, the chemotaxing cells failed to form streams; they moved toward the cAMP source either as single cells or as small flat clumps, like cAMP-unpulsed cells. Taken together, these results strongly suggest that *srfB*-null cells are impaired in cAMP signal relay, not however in cAMP sensing.

Despite these problems in chemotaxis and cell adhesion, *srfB*$^-$ cells formed aggregates on nitrocellulose filters or agar 2 h earlier than wild type cells, as mentioned above. No streams were observed and aggregation resulted from rapid formation of small clumps that grew larger, possibly by accretion with other small clumps.

Post-aggregative multicellular development was completed by the *srfB* mutant strain and fruiting bodies were formed 24 h after starvation (Fig. 8A). However, the mutant strain showed an increased tendency to form migratory slugs under these conditions, that favor direct culmination in WT strains (18–22 h of development in Fig. 8A). Slug formation was also observed when cells were grown on bacteria and resulted in the presence of numerous trails left on the plates by migrating slugs (data not shown). Developmental timing was also analyzed by the expression of the prestalk-specific *ecmB* and the prespore-specific *cotD* genes. The expression

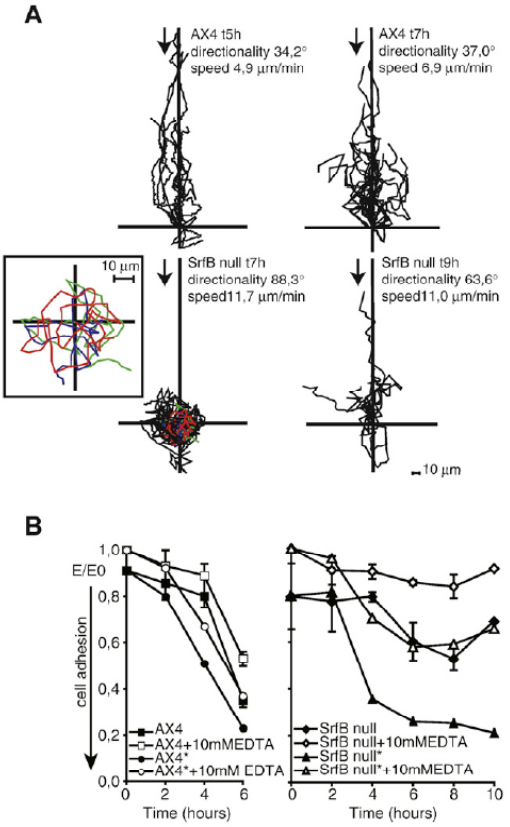


Fig. 7. Chemotaxis and cell adhesion of wild type and *srfB* mutant cells. Panel A: Wild type (AX4) or *srfB* mutant (*srfB*$^-$) cells were starved under shaking for the time indicated, washed and plated on glass-based dishes. The movement of the cells towards cAMP, diffusing from a micropipette, was recorded and analyzed using ImageJ software. Migration speed and directionality of cells was measured as described in Materials and methods. Arrows indicate the source of the cAMP gradient. The insert is an enlargement of three representative tracings (in colors), to better show cell movement around, or close to, the cell axis. The distance in µm from the origin is also indicated. Panel B: Determination of EDTA-sensitive and EDTA-stable adhesion for AX-4 and *srfB*$^-$ cells. AX4 and *srfB*-null cells were incubated under shaking. At the times indicated in the abscissa, AX4 (on the left) and *srfB*$^-$ (on the right) cells were washed, resuspended at a final concentration of 1×10^7 per ml and incubated with (open symbols) or without (closed symbols) 10 mM EDTA in 0.2 ml volume cuvettes. Samples containing control and mutant cells treated with pulses of 20 nM cAMP every 6 min, starting at time 0 are marked with asterisks. Aggregation was measured by using the light scattering assay as described in Materials and methods. The light scattering assay measures unscattered light (E=extinction) at equilibrium, i.e. after 40 to 60 min of incubation. Unscattered light is high when cells are single and diminishes with increasing cell clumping. The values are normalized for E_0 (the value of EDTA-treated, totally dissociated cells at time 0). A value of 1 corresponds to single cells and lower E/E_0 values correlate with formation of larger and more compact aggregates. Mean values, with error bars, of two separate experiments in duplicate are shown.

of both genes was induced 2 h earlier in the *srfB*$^-$ strain (Fig. 8B). However, *ecmB* induction at culmination occurred at the same time (20 h) in *srfB*$^-$ and WT strains. A similar change in the pattern of expression was observed for the *ampA* gene, which was induced 2 h earlier in the mutant and maintained high-expression level for a longer time than the wild type strain (Fig. 8B). Spores formed by the *srfB* mutant strain were more heterogeneous in size and shape than WT spores but no difference in viability was observed.

Actin cytoskeleton-related functions in growing cells

SrfB$^-$ cells are larger and more heterogeneous in size than WT cells when growing on plastic surfaces. The mutant strain presented a significantly higher proportion of cells with two or more nuclei, as compared with WT cells (Fig. 9A). Mutant cells over-expressing *srfB* partially reverted the larger proportion of multi-nucleated cells (Fig. 9B).

Since impaired actin cytoskeleton function might produce defects in cytokinesis, which could result in larger cells, another actin cytoskeleton-dependent process, macropinocytosis, was investigated. Incorporation of media containing FITC-dextran was faster by *srfB* mutant cells than by WT cells (Fig. 9C). The difference in ingestion rate was of 1.6 times. *SrfB*$^-$ cells that over-express *srfB* showed a macropinocytosis rate similar to the one of WT cells (Fig. 9C).

Analyses of srfB-dependent gene expression

DNA microarrays containing 9300 probes (for approximately 8600 different genes) were hybridized to identify genes whose expression was dependent on *srfB*. RNAs were obtained from WT and *srfB*$^-$ strains at early mound stage. A large number of genes are differently expressed in the *srfB*$^-$ and WT strains. Using a cutoff of $p<0.001$, a total of 276 were over-expressed in the *srfB* mutant strain and 301 were under-expressed. Among them, 53 genes were over-expressed and 157 under-expressed more than 2 fold in the mutant strain. Since only one time point was used, the microarray data do not allow one to distinguish whether the differences observed between strains are due to altered timing or altered expression level, and therefore they have to be considered preliminary. For this reason, gene expression was analyzed by Northern blot for some representative genes.

The genes coding for ponticulin (*ponA*) and cofilin 2 (*cofC*), involved in actin cytoskeleton structure and regulation, are over-expressed more than 2 fold in the mutant. Northern blot analyses showed that the ponticulin-coding gene, *ponA*, was expressed during proliferation and repressed after starvation. The expression of this gene was maintained for a longer time in the *srfB*$^-$ strain (Fig. 10). Similarly, the gene coding for cofilin 2 (*cofC*) was induced earlier and to higher levels in the mutant cells (Fig. 10). Mutant cells expressing *srfB* showed a pattern of *ponA* and *cofC* expression more similar to that of wild type cells than to the mutant ones (*srfB*$^-$::*srfB*2, Fig. 10).

There are 14 genes coding for lysosomal proteins and 24 genes coding for ribosomal proteins that are expressed at levels at least 2 fold lower in the mutant than in the WT strain. For

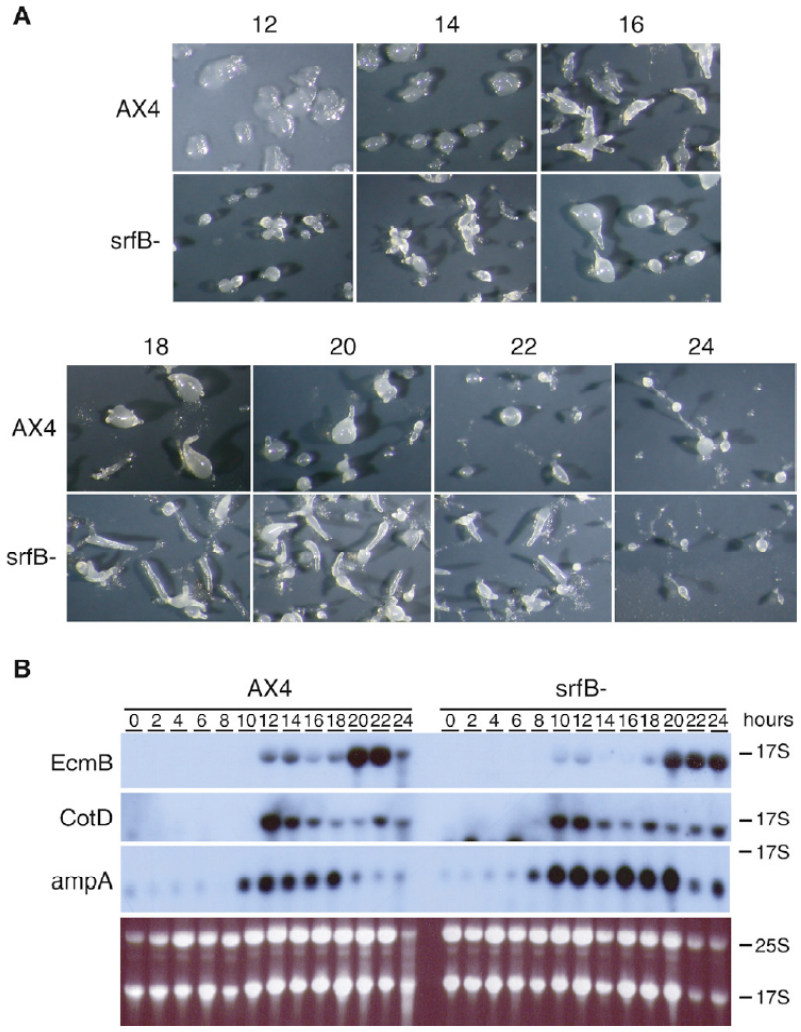


Fig. 8. Post-aggregation development of wild type and *srfB* mutant strains. Panel A: Wild type (AX4) or *srfB* mutant (*srfB*$^-$) cells were deposited on nitrocellulose filters to induce multicellular development. The structures found at middle and late stages of development (12, 14, 16, 18, 20, 22 and 24 h) are shown. Panel B: RNA was obtained from wild type (AX4) and *srfB* mutant (*srfB*$^-$) cells during proliferation (0) or at different stages of development on nitrocellulose filters (2–24 h). The three upper panels show the hybridization with a probe for the prestalk-specific gene *ecmB*, the prespore-specific gene *cotD* and the *ampA* gene. Ethidium bromide staining of a representative gel is shown in the lower panel. Migration of the ribosomal RNAs is indicated at the right.

example, genes coding for lysozyme (*alyA, B, C*) and pre-procathepsin D (*ctsD*) are some of the more importantly down-regulated genes in the mutant (7 and 10 fold, respectively). Northern blot analyses indicated that lysosomal (*ctsD*, *alyB*) and ribosomal (*rps12*) protein encoding genes are repressed at starvation. Repression is faster and more pronounced in the *srfB*$^-$ strain, in concordance with the lower expression detected with the microarrays (Fig. 10). SrfB expression in mutant cells induced a pattern of *alyB*, *ctsD* and *rps12* expression similar to that of wild type cells.

Some of the differentially expressed genes are regulated at the transition between growth and development, or have a role in development or cell differentiation. Among them, discoidin genes (A, B and C chains) are up-regulated, and two countin genes (*ctnA*, *ctnC*) are down-regulated in the *srfB* mutant. Again these putatively differentially expressed genes were analyzed by Northern blot. The discoidin coding gene *dscA* showed higher expression in *srfB*$^-$ than in WT cells (Fig. 10). The countin coding gene, *ctnA*, was expressed at lower levels in *srfB* mutant cells and repressed at earlier developmental stages

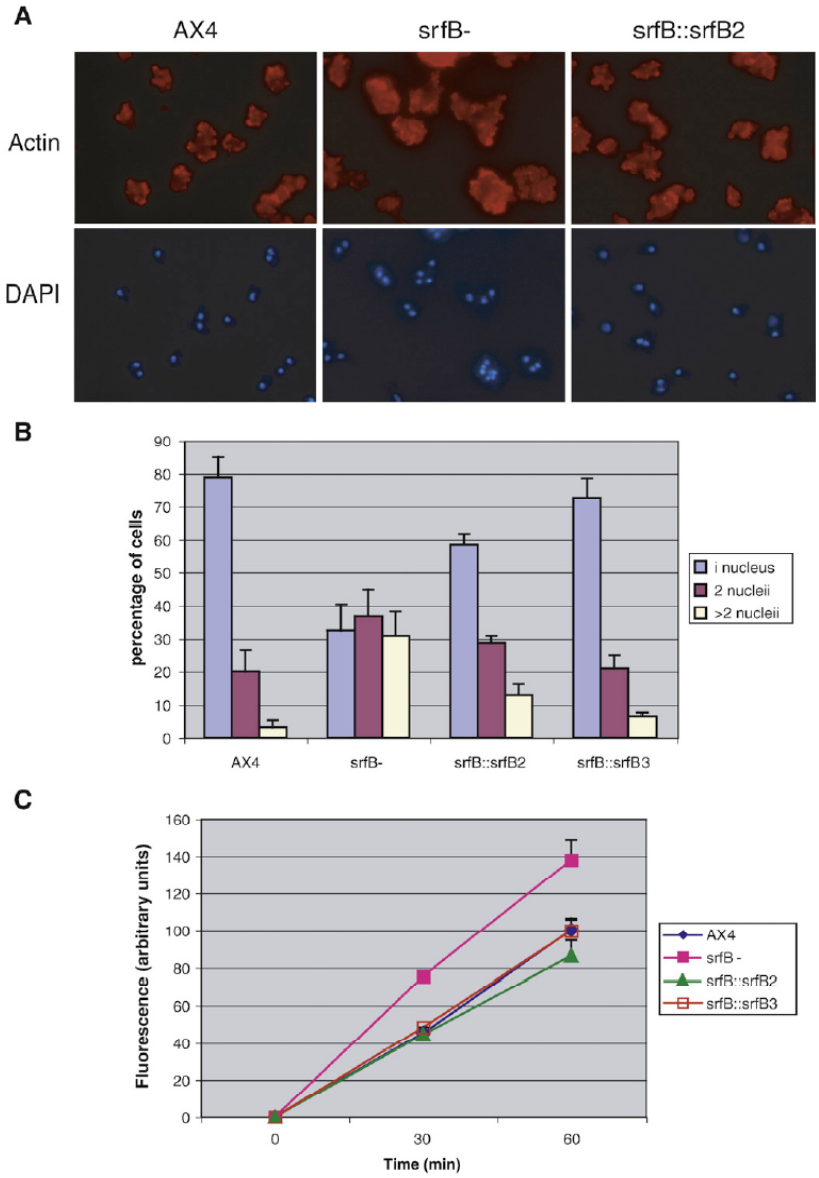


Fig. 9. Study of cytokinesis and macropinocytosis in wild type and *srfB* mutant cells. Panel A: Wild type cells (AX4), *srfB* mutant (*srfB*$^-$) cells or mutant cells that express *srfB* (*srfB*: :*srfB*2) were grown on plastic plates and stained for F-actin (actin) and with the DNA staining reagent 4,6-diamidino-2-phenylindole (DAPI). Panel B: Quantification of the percentage of cells that present 1, 2 or more than 2 nuclei in wild type (AX4) *srfB* mutant (*srfB*$^-$) or mutant cells that express *srfB* (*srfB*: :*srfB*2, *srfB*: :*srfB*3). Mean and standard deviations are represented. Panel C: Pinocytosis was measured by the uptake of FITC-dextran present in the culture media. Mean and standard deviations of wild type (AX4), *srfB* mutant (*srfB*$^-$) cells and mutant cells that express *srfB* (*srfB*: :*srfB*2, *srfB*: :*srfB*3) at 30 and 60 min of incubation are represented.

than in WT cells (Fig. 10). Expression of *srfB* in mutant cells resulted in an increase in *ctnA* expression and a decrease in *dscA* expression after starvation, making expression patterns similar to the wild type ones.

Discussion

Analysis of the *D. discoideum* genome detected the existence of two SRF-homologous genes, *srfA*, previously characterized,

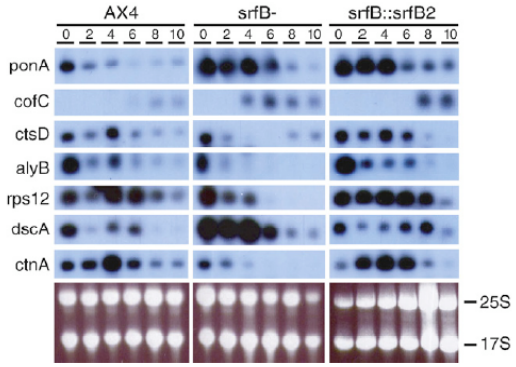


Fig. 10. Developmental expression of *srfB*-regulated genes. RNA was obtained from wild type (AX4), *srfB* mutant (*srfB*$^-$) cells and mutant cells expressing *srfB* (*srfB*: :*srfB*2) during proliferation (0) or at different developmental stages (2–10). The expression of the genes coding for actin cytoskeleton regulatory proteins (Ponticulin (*ponA*), cofilin 2 (*cofC*)), lysosomal proteins (prepro-cathepsin D (*ctsD*), lysozyme (*alyB*)), ribosomal proteins (ribosomal protein S12 (*rps12*)), and genes involved in multicellular development (Discoidin (*dscB*), countin (*ctnA*)) is shown. Ethidium bromide staining of representative gels is shown in the lower panel. Migration of the ribosomal RNAs is indicated to the right of each panel. The mRNAs corresponding to the genes analyzed in this figure migrated faster than the 17S ribosomal RNA.

and *srfB*, that is the subject of this study. The animal species whose genome sequence is known, have a single SRF homologous gene, while *S. cerevisiae* contains two (*mcm1* and *argR1*) and plants possess numerous genes (Theiben et al., 1996). Phylogenetic analyses indicate that *D. discoideum* and *S. cerevisiae* SRF-homologous genes were originated by gene duplication after the divergence of animal, fungi and Dictyostelids.

SrfB is expressed during proliferation and multicellular development under the control of different promoter regions. The distal promoter directs transcription in vegetative cells and in cells scattered in the mound and finger structures. The two more proximal promoters direct expression in vegetative cells, cells scattered at the mound stage and prestalk (PstAB region) and stalk cells in developing structures. However, some regulatory regions controlling the activity of these promoters must be dispersed in the intergenic region because reporter vectors that contain this complete region recapitulate the pattern of expression of the three promoters, directing higher expression levels than any of them. It is remarkable that the 5′UTR regions (up to 817 bp) and the introns present in the 5′UTR (1066 and 1509 bp long) are unusually large in comparison to other *D. discoideum* genes, that present a mean intron size of 146 bp (Eichinger et al., 2005).

The *srfB* homologous gene, *srfA*, is also transcribed from several promoters that direct expression to different regions of the structure, although expression patterns are very different for both genes (Escalante et al., 2001).

The biological functions in which *srfB* is involved have been studied by the generation of a mutant strain, where part of the MADS-box coding region of the gene has been substituted by a blasticidin-resistance gene. *SrfB* mutant cells showed several defects both during growth and development, consistent with the complexity of the promoter.

During proliferation *srfB*$^-$ cells showed alterations in cytokinesis and pinocytosis. They also showed impaired migration and adhesion of the rear end to the substrate during early development. These defects could be indicative of impaired actin cytoskeleton function.

Mutant cells also displayed an altered starvation response, in comparison to WT cells. *SrfB* mutant cells seem to respond to starvation faster than WT cells. Aggregates are formed on nitrocellulose or agar about 2 h earlier in *srfB*$^-$ than in WT cells. Some early aggregation genes, such as those coding for the cAMP receptor (*carA*) or adenylyl cyclase A (*acaA*) are also induced about 2 h earlier in the mutant. In addition, some of the genes that are quickly repressed after starvation, such as those coding for ribosomal proteins (Ken and Singleton, 1994; Agarwal et al., 1999) and lysosomal enzymes (Bush et al., 1994) are down-regulated in the mutant. Discoidin is also induced earlier and to higher levels in the mutant. The developmental timing of expression of these genes is reverted when srfB is expressed in the mutant strain. Complex changes in gene expression have been described for *D. discoideum* cells upon starvation (Iranfar et al., 2001). The data obtained for the mutant indicate that *srfB* could be involved in these changes at the proliferation/development transition.

In apparent contrast to these observations, *srfB* mutant cells showed a developmental delay when incubated in shaken cultures, because chemotaxis to cAMP, the main process that directs cell aggregation, and acquisition of EDTA-stable cell adhesion are highly impaired. This apparent contradiction can be solved by assuming that early aggregate formation on nitrocellulose filters or agar is mainly due to clumping of cells by random collision. In favor of this interpretation is the increased EDTA-labile adhesion measured in the mutant that may explain rapid formation of small clumps, in the absence of chemotaxis. Stream formation, which is typical of chemotaxing cells, is not observed on agar. Aggregate formation could make possible subsequent steps of development on nitrocellulose filters or agar.

Chemotaxis studies with cAMP diffusing from a microcapillary show that mutant cells are impaired in migration towards cAMP. Despite their increased motility and a reduced though clear orientation ability, the *srfB*$^-$ cells do not become polarized and do not migrate or migrate poorly towards the cAMP source. Instead, the cells move around their axis, remaining mainly attached with their rear end to the glass substrate.

Developmentally regulated EDTA-stable cell adhesion is also strongly delayed under shaking. EDTA-stable adhesion is dispensable for aggregation on nitrocellulose filter or agar, though contributing to optimal aggregation and becoming essential under more stringent cell–substrate interactions (Harloff et al., 1989; Ponte et al., 1998). Nevertheless, EDTA-stable adhesion is a good marker of cAMP-dependent aggregation-specific gene expression (Gerisch, 1987).

Remarkably, cAMP pulsing restores EDTA-stable adhesion, indicating that the *srfB*$^-$ null mutant is able to respond to cAMP, but is defective in generating endogenous cAMP signals, which

are necessary for expression of the csA glycoprotein and other genes required for aggregation (Mehdy and Firtel, 1985; Gerisch, 1987; Iranfar et al., 2003). This conclusion is further supported by the finding that cAMP-pulsed cells fail to form streams, when stimulated by cAMP diffusing from a micro-capillary, indicating a defect in cAMP signal relay. Although polarization and cell orientation toward the cAMP source were strongly improved by cAMP pulses, a third defect of the mutant, namely the strong attachment of the cell uropod to the substratum or to small clumps was rescued only minimally, resulting in a modest increase in the chemotaxis index.

Understanding the molecular basis of such a complex phenotype requires additional experiments that will be done in the future. It is important to find out if the defect in cAMP signaling is upstream or downstream of the heterotrimeric G protein and whether misregulation is at the level of adenylyl cyclase or cAMP phosphodiesterase activity or in any other component of the cAMP signalling. A protein involved in cAMP wave propagation, the phophodiesterase inhibitor PDI, is for example overexpressed 2.5 fold in the mutant (data not shown).

Correlating the defect with *srfB*-gene activity ultimately requires identification of the cis-acting target sites at DNA level. A few conclusions can be, however, drawn from these experiments: all phenotypic defects of the mutant during aggregation, with exception of the unusually strong cell adhesion to the substrate, can be explained with defective cAMP signaling. Although we cannot exclude that SrfB may directly regulate expression of one or more genes involved in cAMP signaling, it is more likely that the defect in cAMP signaling is secondary to misregulation of genes involved in the transition from growth to development. As shown by the gene expression data, several genes involved in this transition are indeed either down- or over-expressed in the mutant.

We have no explanation for the localized strong adhesion of the cells at their rear end to the glass substrate, and to our knowledge this is the first time that such a phenotype has been described. Overexpression of ponticulin and cofilin 2 could in part explain this phenotype. Ponticulin is an integral plasma membrane protein that binds actin filaments to the membrane. Cells lacking ponticulin aggregate sooner than WT cells (Hitt et al., 1994). Cofilin 2 stimulates turn-over of actin filaments. This protein is located at the cell–substrate adhesion sites (Aizawa et al., 2001). Over-expression of the related cofilin-1 gene produces enhancement of spontaneous cell motility (Aizawa et al., 1996), as also observed in the srfB mutant. Over-expression of these proteins could explain cell spreading and increased adhesion to the substrate, but not the localized cell attachment at the uropod.

In addition, two genes coding for countin are strongly down-regulated in the mutant (ctnA-5, 6 fold; ctnC-5, 4 fold and Fig. 10). Countin participates in the determination of the size of the structures by regulating cell–cell adhesion (Roisin-Bouffay et al., 2000). Countin mutant cells also show decreased F-actin level, and increased cell motility and cell–cell adhesion (Tang et al., 2002). Therefore, the decrease in ctnA and ctnC expression could also contribute to some of the defects observed in cell motility and cell–cell adhesion in the *srfB* mutant.

D. discoideum development continues from aggregation through two alternative pathways. In the presence of ammonia the structures lay on their sides to originate a migratory structure (slug) (Hashimoto and Matsui, 1988). If ammonia does not accumulate, the fruiting body is directly formed at the site of aggregation (Kristen et al., 2005). The mechanisms that regulate slug formation versus culmination are not well understood but the cells at the tip of the structure seem to have a regulatory role in this process (Chisholm and Firtel, 2004). *SrfB* mutant structures show a marked tendency to form slugs under conditions that favor direct culmination of WT structures. The expression of *srfB* in the prestalk PstAB cells, and in the first cells that migrate towards the substrate to form the stalk, might indicate that *srfB* could participate in the process of initiation of culmination.

SRF regulates the expression of many components of the actin cytoskeleton and contractile apparatus in animals, including actin and myosin isoforms (Miano et al., 2007). However, no changes in the expression of actin or most of myosin isoforms have been detected in the microarray analyses of the *srfB* mutant. Only an actin-related protein and, at lower levels, myoI and the essential and regulatory myosin light chains show changes of expression in the mutant (data not shown). Regulation of actin and myosin expression is, however, complex in *D. discoideum* due to the existence of 30 genes coding for actin (Eichinger et al., 2005), 13 for myosin heavy chain and 4–6 for myosin light chains (Kollmar, 2006). Analyses of the nucleotide sequence detected the existence of possible SRF-binding sites in the putative promoter region of the *actin15*, Myosin A and *mlcR* genes. The regulatory function of these putative SRF binding sites is presently under investigation. However, no consensus SRF-binding site (CCA/T_6GG) was identified in the analyses of the putative promoter region of the genes whose expression is altered in the *srfB*$^-$ strain during development. This observation further supports the hypothesis mentioned above for the cAMP-dependent gene expression, namely that the regulation by SrfB could be indirect, through the regulation of the expression of other transcription factors at the transition from growth to development. The analyses of the promoter region of 24 SrfA-dependent genes did not show the presence of any consensus SRF-binding site either. Because of this reason, the possibility that the SRF-binding site could have changed should be considered and a functional analysis of these promoters will be required to ascertain their transcriptional regulation by SrfB.

Globally considered, the data obtained indicate that *srfB* plays important roles in the regulation of the proliferation/development transition and in the initiation of culmination during fruiting body formation. Future studies, directed at identifying the regulated target genes, may help in furnishing a mechanistic interpretation of *srfB* gene activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.026.

References

Adachi, H., Hasebe, T., Yoshinaga, K., Ohta, T., Sutoh, K., 1994. Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. Biochem. Biophys. Res. Commun. 205, 1808–1814.

Agarwal, A.K., Parrish, S.N., Blumberg, D.D., 1999. Ribosomal protein gene expression is cell type specific during development in *Dictyostelium discoideum*. Differentiation 65, 73–88.

Aizawa, H., Sutoh, K., Yahara, I., 1996. Overexpression of cofilin stimulates bundling of actin filaments, membrane ruffling, and cell movement in *Dictyostelium*. J. Cell Biol. 132, 335–344.

Aizawa, H., Kishi, Y., Iida, K., Sameshima, M., Yahara, I., 2001. Cofilin-2, a novel type of cofilin, is expressed specifically at aggregation stage of *Dictyostelium discoideum* development. Genes Cells 6, 913–921.

Alberti, S., Krause, S.M., Kretz, O., Philippar, U., Lemberger, T., Casanova, E., Wiebel, F.F., Schwartz, H.L., Frostscher, M., Schutz, G., Nordheim, A., 2005. Neuronal migration in the murine rostral migratory stream requires serum response factor. Proc. Natl. Acad. Sci. U. S. A. 102, 6148–6153.

Alvarez-Buylla, E.R., Pelaz, S., Liljegren, S.J., Gold, S.E., Burgeff, C., Ditta, G.S., Ribas de Pouplana, L., Martinez-Castilla, L., Yanofsky, M.F., 2000. An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. Proc. Natl. Acad. Sci. U. S. A. 97, 5328–5333.

Arigoni, M., Bracco, E., Lusche, D.F., Kae, H., Weeks, G., Bozzaro, S., 2005. A novel *Dictyostelium* RasGEF required for chemotaxis and development. BMC Cell Biol. 6, 43.

Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., Nordheim, A., 1998. Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J. 17, 6289–6299.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J.R. Stat. Soc. Ser B 57, 289–300.

Boxer, L.M., Prywes, R., Roeder, R.G., Kedes, L., 1989. The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response factor. Mol. Cell. Biol. 9, 515–522.

Bozzaro, S., 2006. Assaying cell–cell adhesion. In: Eichinger, L., Rivero, F. (Eds.), *Dictyostelium discoideum* Protocols, vol. 346. Humana Press, Totowa, pp. 449–468.

Bozzaro, S., Merkl, R., Gerisch, G., 1987. Cell adhesion: its quantification, assay of the molecules involved, and selection of defective mutants in *Dictyostelium* and *Polysphondylium*. In: Spudich, J.A. (Ed.), Methods in Cell Biology, vol. 28. Ac. Press, Orlando, FL, pp. 359–385.

Bush, J., Richardson, J., Cardelli, J., 1994. Molecular cloning and characterization of the full-length cDNA encoding the developmentally regulated lysosomal enzyme beta-glucosidase in *Dictyostelium discoideum*. J. Biol. Chem. 269, 1468–1476.

Chisholm, R.L., Firtel, R.A., 2004. Insights into morphogenesis from a simple developmental system. Nat. Rev., Mol. Cell Biol. 5, 531–541.

Detterbeck, S., Morandini, P., Wetterauer, B., Bachmair, A., Fischer, K., MacWilliams, H.K., 1994. The 'prespore-like cells' of *Dictyostelium* have ceased to express a prespore gene: analysis using short-lived beta-galactosidases as reporters. Development 120, 2847–2855.

Eichinger, L., Pachebat, J.A., Glockner, G., Rajandream, M., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., et al., 2005. The genome of the social amoeba *Dictyostelium discoideum*. Nature 435, 43–57.

Escalante, R., Sastre, L., 1998. A serum response factor homolog is required for spore differentiation in *Dictyostelium*. Development 125, 3801–3808.

Escalante, R., Sastre, L., 2006. Investigating gene expression: in situ hybridization and reporter genes. In: Eichinger, L., Rivero, F. (Eds.), *Dictyostelium discoideum* Protocols, vol. 346. Humana Press, Totowa, NJ, pp. 230–247.

Escalante, R., Vicente, J.J., Moreno, N., Sastre, L., 2001. The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of *Dictyostelium* development. Dev. Biol. 235, 314–329.

Escalante, R., Yamada, Y., Cotter, D., Sastre, L., Sameshima, M., 2004. The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during *Dictyostelium* sporulation. Mech. Dev. 121, 51–56.

Gentleman, R.C., Carey, V.J., Bates, D.M., Al, E., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Gerisch, G., 1987. Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium*. Annu. Rev. Biochem. 56, 853–879.

Harloff, C., Gerisch, G., Noegel, A.A., 1989. Selective elimination of the contact site A protein of *Dictyostelium discoideum* by gene disruption. Genes Dev. 3, 2011–2019.

Hashimoto, Y., Matsui, K., 1988. Thermotaxis and the role of the *Dictyostelium discoideum* slug tip. Cell Struct. Funct. 13, 189–191.

Hitt, A.L., Lu, T.H., Luna, E.J., 1994. Ponticulin is an atypical membrane protein. J. Cell Biol. 126, 1421–1431.

Iranfar, N., Fuller, D., Sasik, R., Hwa, T., Laub, M., Loomis, W.F., 2001. Expression patterns of cell-type-specific genes in *Dictyostelium*. Mol. Biol. Cell 12, 2590–2600.

Iranfar, N., Fuller, D., Loomis, W.F., 2003. Gene regulation during early development of *Dictyostelium* using genome-wide expression analyses. Eukaryotic Cell 2, 664–670.

Jin, T., Hereld, D., 2006. Moving towards understanding eukaryotic chemotaxis. Eur. J. Cell Biol. 85, 905–913.

Ken, R., Singleton, C.K., 1994. Redundant regulatory elements account for the developmental control of a ribosomal protein gene of *Dictyostelium discoideum*. Differentiation 55, 97–103.

Kollmar, M., 2006. Thirteen is enough: the myosins of *Dictyostelium discoideum* and their light chains. BMC Genomics 7, 183–198.

Kooperberg, C., Fazzio, T.G., Delrow, J.J., Tsukiyama, T., 2002. Improved background correction for spotted DNA microarrays. J. Comput. Biol. 9, 55–66.

Kristen, J.H., Xiong, Y., Dunbar, A.J., Rai, M., Singleton, C.K., 2005. Ammonium transporter C of *Dictyostelium discoideum* is required for correct prestalk gene expression and for regulating the choice between slug migration and culmination. Dev. Biol. 287, 146–156.

Li, S., Czubryt, M.P., McAnally, J., Bassel-Duby, R., Richardson, J.A., Wiebel, F.F., Nordheim, A., Olson, E.N., 2005. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. Proc. Natl. Acad. Sci. U. S. A. 102, 1082–1087.

Maeda, Y., 2005. Regulation of growth and differentiation in *Dictyostelium*. Int. Rev. Cyt. 244, 287–332.

Mehdy, M.C., Firtel, R.A., 1985. A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. Mol. Cell. Biol. 5, 705–713.

Miano, J.M., Ramanan, N., Georger, M.A., de Mesy-Bentley, K.L., Emerson, R.L., Balza, R.O.J., Xiao, Q., Weiler, H., Ginty, D.D., Misra, R.P., 2004. Restricted inactivation of serum response factor to the cardiovascular system. Proc. Natl. Acad. Sci. U. S. A. 101, 17132–17137.

Miano, J.M., X, L., Fujiwara, K., 2007. Serum response factor: master regulator of the actin cytoskeleton and contractil apparatus. Am. J. Physiol.: Cell Physiol. 292, C70–C81.

Pang, K.M., Lynes, M.A., Knecht, D.A., 1999. Variables controlling the expression level of exogenous genes in *Dictyostelium*. Plasmid 41, 187–197.

Pergolizzi, B., Peracino, B., Silverman, J., Ceccarelli, A., Noegel, A., Devreotes, P., Bozzaro, S., 2002. Temperature-sensitive inhibition of development in *Dictyostelium* due to a point mutation in the piaA gene. Dev. Biol. 251, 18–26.

Ponte, E., Bracco, E., Faix, J., Bozzaro, S., 1998. Detection of subtle phe-

notypes: The case of the cell adhesion molecule csA in *Dictyostelium*. Proc. Natl. Acad. Sci. U. S. A. 95, 9360–9365.

Posern, G., Treisman, R., 2006. Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol. 16, 588–596.

Rivero, F., Maniak, M., 2006. Quantitative and microscopic methods for studying the endocytic pathway. In: Eichinger, L., Rivero, F. (Eds.), *Dictyostelium discoideum* Protocols, vol. 346. Humana Press, Totowa, NJ, pp. 423–438.

Roisin-Bouffay, C., Jang, W., Caprette, D.R., Gomer, R.H., 2000. A precise group size in *Dictyostelium* is generated by a cell-counting factor modulating cell–cell adhesion. Mol. Cell 6, 953–959.

Sambrook, J., Frisch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Schratt, G., Philippar, U., Berger, J., Schwarz, H., Heidenreich, O., Nordheim, A., 2002. Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. J. Cell Biol. 156, 737–750.

Shaulsky, G., Loomis, W.F., 1993. Cell type regulation in response to expression of ricin-A in *Dictyostelium*. Dev. Biol. 160, 85–98.

Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 (Article 3).

Smyth, G.K., 2005. Limma: linear models for microarray data. In: Gentleman, V.C.R., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer, New York.

Sun, Q., Chen, G., Streb, J.W., Long, X., Yang, Y., Stoeckert Jr, C.J., Miano, J.M., 2007. Defining the mammalian CArGome. Genome Res. 16, 197–207.

Tang, L., Gao, T., McCollum, C., Jang, W., Vicker, M.G., Ammann, R.R., Gomer, R.H., 2002. A cell number-counting factor regulates the cytoskeleton and cell motility in *Dictyostelium*. Proc. Natl. Acad. Sci. U. S. A. 99, 1371–1376.

Theiben, G., Kim, J.T., Saedler, H., 1996. Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J. Mol. Evol. 43, 484–516.

Treisman, R., 1987. Identification and purification of a polypeptide that binds to the c-*fos* serum response element. EMBO J. 6, 2711–2717.

Treisman, R., 1995. DNA-binding proteins. Inside the MADS box. Nature 376, 468–469.

Zang, S.X., Gras, E.G., Wycuff, D.R., Marriot, S.J., Kadeer, N., Yu, W., Olson, E.N., Garry, D.J., Parmacek, M.S., Schwartz, R.J., 2005. Identification of direct Serum Response Factor gene targets during DMSO induced P19 cardiac cell differentiation. J. Biol. Chem. 280, 19115–19126.

Capítulo 2: *The Dictyostelium discoideum acaA gene is transcribed from alternative promoters during aggregation and multicellular development.*

El AMPc extracelular es una molécula de señalización clave en la regulación de procesos tan importantes como la agregación, la diferenciación celular y la morfogénesis durante el desarrollo pluricelular de la ameba social *Dictyostelium discoideum.* Esta molécula se produce por tres encimas Adenilato ciclasas diferentes, codificadas cada una por los genes *acaA, acrA y acgA*, que se expresan durante diferentes estadios de desarrollo y en diferentes estructuras. Se ha descrito que *acaA* se expresa mayoritariamente en células en agregación. La proteína AcaA se encargaría de sintetizar el AMPc que es necesario para la señalización célula-célula durante este proceso de agregación celular y la formación de los "*streams*". En este trabajo se describe la caracterización de la región promotora del gen *acaA*, mostrando que su transcripción es llevada a cabo por tres promotores alternativos. El promotor distal, Promotor 1, se activa durante la agregación. El Promotor 2 es activo en células situadas en la parte posterior de las estructuras, con una distribución similar a la de las células pre-espora y, en estadios más avanzados del desarrollo, en el esporangio. El Promotor 3 es activo en células situadas en la región del *"tip"* de las estructuras en fases migratorias de desarrollo (*"slug"*). Posteriormente es activo en las células que forman el tallo de las estructuras que están culminando. El fragmento que contiene los tres promotores dirige la expresión en las mismas regiones. Estos resultados obtenidos usando vectores que expresan *lacZ* se corroboraron por hibridación *in situ* utilizando sondas específicas de cada uno de los tres ARNm de codifican AcaA. El análisis de la expresión de los ARNm por RT-PCR cuantitativa, con oligonucleótidos específicos para cada uno de los tres transcritos, también demostró que tienen diferentes patrones de expresión.

La existencia de un promotor específico de agregación puede ser asociada con el uso del AMPc como molécula quimioatrayente y esto sólo ocurre en algunas especies de Dictiostélidos. AcaA sería el tercer gen implicado en la señalización por AMPc que poseyera una región distal promotora que dirige específicamente la expresión del gen en agregación. La expresión durante los estadios más avanzados del desarrollo indica que la Adenilato ciclasa A podría jugar un papel, más importante del que se había considerado hasta ahora, durante el desarrollo posterior a la agregación.

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The *Dictyostelium discoideum acaA* Gene Is Transcribed from Alternative Promoters during Aggregation and Multicellular Development

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Abstract

Background: Extracellular cAMP is a key extracellular signaling molecule that regulates aggregation, cell differentiation and morphogenesis during multi-cellular development of the social amoeba *Dictyostelium discoideum*. This molecule is produced by three different adenylyl cyclases, encoded by the genes *acaA*, *acrA* and *acgA*, expressed at different stages of development and in different structures.

Methodology/Principal Findings: This article describes the characterization of the promoter region of the *acaA* gene, showing that it is transcribed from three different alternative promoters. The distal promoter, promoter 1, is active during the aggregation process while the more proximal promoters are active in tip-organiser and posterior regions of the structures. A DNA fragment containing the three promoters drove expression to these same regions and similar results were obtained by in situ hybridization. Analyses of mRNA expression by quantitative RT-PCR with specific primers for each of the three transcripts also demonstrated their different temporal patterns of expression.

Conclusions/Significance: The existence of an aggregation-specific promoter can be associated with the use of cAMP as chemo-attractant molecule, which is specific for some *Dictyostelium* species. Expression at late developmental stages indicates that adenylyl cyclase A might play a more important role in post-aggregative development than previously considered.

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Introduction

Intercellular communication plays a central role in multi-cellular development, coordinating processes such as cell migration, proliferation and differentiation that are the basis for morphogenesis. One of the simplest organisms where these processes have been studied is the social amoeba *Dictyostelium discoideum* (for recent reviews, [1,2]). These organisms live as individual amoebe in forest soils. Upon starvation, up to 100.000 cells aggregate together and form a fruiting body, composed of a basal disk, a stalk and, on top of it, a sorus.

D. discoideum fruiting body formation is one of the more primitive examples of multi-cellular development but, even so, is a complex process that is tightly regulated [3]. Cell aggregation is mediated by cAMP in *D. discoideum* [4]. Cells in the aggregation fields are able to move towards increasing cAMP concentrations and to secrete cAMP, so that the signal gets amplified [5]. Upon aggregation, mounds are formed where cells differentiate in two main alternative pathways: prestalk or prespore cells. Prestalk cells associate together and move to the upper part of the mound, where they emerge as a tip [6,7], while prespore cells remain in the lower part of the mound [8]. The tip region acts as an organizing center during later development [9,10]. Culmination takes place when prestalk cells, located in the tip, migrate towards the substrate through the mass of prespore cells, elongate and synthesize a cellulose outer layer. As a consequence, prespore cells are raised from the substrate to form a sorus, and complete the differentiation process.

Several signaling molecules coordinate *D. discoideum* development but, among them, cAMP plays a central role (reviewed in [1,11]). As mentioned above, extracellular cAMP first mediates aggregation [4]. Later on, cAMP secreted from the tip is required for prestalk and prespore cells sorting in the mound [12]. Extracellular cAMP at a high, constant level is required for in vitro prespore and prestalk differentiation [13,14], inducing or repressing the expression of cell-type specific genes [15]. The decision of initiating culmination is also dependent on extracellular cAMP, that activates the STATa transcription factor at the tip organiser region, in the anterior of the slug, initiating a regulatory cascade that proceeds through activation of the CudA transcription factor [16,17] and the tip-organiser-specific expression of their targets genes, such as *expl7*[18]. Finally, high extracellular cAMP

levels are required for spore differentiation [19], for GSK-3 mediated inhibition of stalk cell formation [20,21] and for regulation of spore germination [22]. Additional signaling molecules contribute to coordinate some of these processes. For example, stalk cell differentiation is induced by the chlorinated hexaphenone DIF-1, secreted by prespore cells, in addition to cAMP, as shown in in vitro experiments [23]. Similarly, spore differentiation is quickly induced by SDF1 and SDF2, produced by prestalk cells, in the presence of high levels of extracellular cAMP [24]. cAMP also plays an important role as an intracellular signaling molecule. For example, cAMP-dependent activation of protein kinase A is required for cAMP signaling at initiation of development [25] and spore differentiation [26,27].

The extensive use of cAMP as signaling molecule requires a tight control of its synthesis and degradation (reviewed by [11,28]). Synthesis is catalyzed by three different adenylyl cyclases, encoded by the genes *acaA*, *acrA* and *acgA*. Degradation is carried out by both extracellular phosphodiestarases, such as PDE, and intracellular ones, such as RegA or PdeE. The expression of these enzymes is regulated during development. In addition, their activity is regulated by extracellular signals, which allows precise control of intra- and extracellular cAMP levels through development [28].

Adenylyl cyclase A is a development-specific enzyme whose synthesis is induced during aggregation [29]. The generation of mutant strains has shown that *acaA* is required for this process [29]. This absolute requirement has impaired the study of the possible involvement of this enzyme during post-aggregative development, although *acaA* expression at the tip of early culminant structures has been described [17].

Adenylyl cyclase B, encoded by the *acrA* gene, is expressed at low levels in proliferating amoebas but its expression is strongly induced from 6 hours of development in prestalk cells [30,31]. Mutant strains form normal structures up to the slug stage but *acrA* is required for culmination and terminal differentiation of the spores [31].

Expression of adenylyl cyclase G, encoded by the *acgA* gene, is induced after 12 hours of development in prespore cells and greatly increased during spore differentiation [29,32]. AcG activity is regulated by osmolarity of the external media [22] and this enzyme is required to avoid spore germination inside the sorus. It has been recently described that AcG homologous enzymes play similar roles in other *Dictyostelium* species during cyst formation [33].

The analysis of the adenylyl cyclases described above indicates that the three enzymes play complementary roles during development. However, the present picture does not seem to be complete. Most of the developmental functions of the three enzymes have been deduced from the phenotype of single [29] [22] [31] or double mutant strains [32] and early developmental defects can preclude the observation of later ones, as can be the case for *acaA* gene mutants, that are blocked at aggregation. In addition, it has been shown that adenylyl cyclase genes regulate each others' expression [32], which could alter their function in mutant strains, as compared with wild-type ones. As a consequences, the function played by each or these enzymes in processes such as prespore and prestalk differentiation, sorting of prestalk and prespore cells in the mound and tip formation is not well determined at the present time.

In this article the structure of the *acaA* promoter region has been studied, showing that this gene is transcribed from three alternative promoters. The use of alternative promoters specific for different developmental stages or cell types has been previously described for some *D. discoideum* genes [34,35,36]. In the case of the *acaA* gene, the existence of alternative promoters allows expression during aggregation and. later on, in the tip-organiser and posterior regions of the structures. These results indicate that the spatio-temporal pattern of *acaA* expression is broader than previously considered and suggest the possibility that *acaA* might be involved in the regulation of several developmental processes, in addition to its well known role in aggregation.

Methods

Cell culture, transformation and development

D. discoideum cells were cultured in HL-5 media. Cells were transformed by electroporation as described by Pang et al. [37]. Transformed cells were selected by treatment with neomycin (G418). Filter development was induced by spreading $0.6\text{-}1.2\times10^6$ cells/cm^2 on Nitrocellulose filters (Millipore Co., Badford, MA, USA) [38].

Rapid amplification of cDNA ends

RNA was isolated from AX4 cells at proliferation or after 8 hours of multicellular development. The SMART^{TM} cDNA amplification kit from Clontech (Clontech Laboratories, Inc, Montain View, CA, USA) was used for amplification of the 5′ untranslated region of the *acaA* mRNA according to the manufacturer's instructions. The oligonucleotide 5′-GGAGATCTACCACCACCATTTCCATCATG-3′, complementary to nucleotides 90 to 110 of the *acaA* coding region, was used as primer in these experiments. Amplification products were cloned in the pGEMT-Easy cloning vector (Promega Co, Madison, WI, USA) and the insert of at least 10 different colonies of each product were sequenced.

Construction of reporter vectors

The three *acaA* promoter regions were amplified by PCR from *D. discoideum* genomic DNA and cloned in the reporter vector PsA-ialphaGal [39] in substitution of the XbaI/BglII PsA promoter fragment. Oligonucleotides 5′-GGTCTAGACTTGATGAGTGGCCAAAACC-3′ and 5′-GGAGATCTATTTTTTAAAGATCCAAGAATTCGTATC-3′, that amplified the -3990 to -2472 genomic region, were used to isolate promoter 1 region. The antisense oligonucleotide included and ATG initiation codon cloned in frame with the *lacZ*-coding region. Oligonucleotides 5′-GGTCTAGAGTTTTTAGATACGAATTCTTGGATC-3′ and 5′-GGAGATCTCATTTACAAAGATATATTTATGAAGTGAGG-3′ amplified the −2500 to −1483 genomic region corresponding to Promoter 2. An ATG in frame initiation codon was also included in the antisense oligonucleotide. Oligonucleotides 5′-GGTCTAGACCTCACTTCATAAATATATCTTTG-3′ and 5′-GGAGATCTACCACCACCATTTCCATCATG-3′, that amplified the −1284 to 110 region were used to amplify promoter 3 region. This fragment included a region coding for the 37 N-terminal AcA aminoacids that were cloned in frame with the β-galactosidase protein. The complete promoter region was cloned in two steps. Promoters 1 and 2 were first cloned together using an internal XhoII site. To incorporate Promoter 3 to this construct a longer fragment (nucleotides −1838 to 110) was generated by PCR using oligonucleotides 5′-GGTCTAGAACCACATTTGTGTGAATTTGATTG-3′ and 5′-GGTCTAGACTTGATGAGTGGCCAAAACC-3′. This fragment was added to the Promoter1+promoter2 fragment using an internal NdeI site.

Histochemistry and determination of β-galactosidase acivity

Cells transformed with the different reporter vectors were allowed to develop on Nitrocellulose filters for the periods or time

indicated in each experiment. Structures were fixed, permeabilized and β-galactosidase activity was detected by hydrolysis of the X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) as previously described [40]. Spores were collected from structures developed on Nitrocellulose filters for 24 hours, fixed and permeabilized before detection of β-galactosidase activity as previously described [40]. β-galactosidase activity was also determined in extracts obtained at different developmental times. 2×10^7 cells were developed on Nitrocellulose filters, collected and lysed in Z Buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4, 10 mM KCl; 1 mM $MgSO_4$, pH: 7.0) containing 0.2% NP40 (Nonidet P40). The enzymatic activity was determined by incubation of the extracts in Z Buffer containing 0.88 mg/ml of the ONPG (2-Nitrophenyl β-D-galactopyranoside) substrate. The amount of ONPG hydrolyzed was estimated by optical absorption at 410 nm and normalized to the amount of protein present in each sample.

Determination of mRNA levels by quantitative RT-PCR

RNA was isolated from 2×10^7 cells, either at growth or after development on Nitrocellulose filters for the times indicated in each experiment, using the TRI reagent (Sigma-Aldrich, Inc, St Louis, MO, USA) according to the manufacturer's instructions. cDNAs were generated from 1 µg of total RNA using gene-specific oligonucleotides as primers. cDNAs were used as substrates for quantitative real-time PCR reactions using as primers the oligonucleotides used for cDNA synthesis and a second oligonucleotide from the upstream region of each transcript. In the case of the acaA mRNAs, the oligonucleotide 5′-GGAGATCTACCAC-CACCATTTCCATCATG-3′, complementary to nucleotides 90 to 110 of the gene, encoded in Exon 2, was used for cDNA synthesis and as reverse primer for PCR amplification. The oligonucleotides 5′-CGTTTTTGATACGAATTCTTGGATC-3′ (nucleotides −2507 to −2483), 5′-CCTCACTTCATAAATA-TATCTTTG-3′ (nucleotides −1284 to −1261) and 5′-CTAG-TAAAATTAATTTGTTGTACC-3′ (nucleotides −459 to −436) were used as forward primers for amplification of the cDNAs corresponding to mRNAs 1, 2 and 3, respectively. The oligonucleotide 5′-GGCATCTAGCTCACCAATG-3′ (nucleotides 3 to 21) was used as forward primer for amplification of a region of the cDNAs contained in Exon 2 that is common to the three mRNAs. A region of the large mitochondrial ribosomal RNA was amplified as a loading control using the oligonucleotides 5′-CACTTTAATGGGTGAACACC-3′ (used for reverse transcription and as reverse PCR primer) and 5′-GGGTAGTTT-GACTGGGGCGG-3′ (forward PCR primer). The iQ5 Real Time PCR Detection System (Bio-Rad Lab. Inc., Hercules, CA, USA) was used in these experiments. PCR products were labeled with Sybr-green using the iQ™SYBR®Green Supermix (Bio-Rad) reaction mix following the manufacturer's instructions. The final volume of the reaction was of 20 µl, using a 0,16 µM concentration of each primer. PCR conditions were as follows: 95°C, 3 m; (95°C, 10 s; 58°C, 30 s; 68°C, 50 s)×40 for mRNA1 and Exon 2 expression; 95°C, 3 m; (95°C, 10 s; 60°C, 30 s; 72°C, 50 s)×40 for mRNA2 and 95°C, 3 m; (95°C, 10 s; 54°C, 30 s; 68°C, 50 s)×40 for mRNA3. The data, obtained in duplicates, were analyzed using the iQ5 Optical system software, version 2.0 (Bio-Rad).

In situ hybridization and probe labelling

Whole-mount *in situ* hybridization of developmental structures was performed according to the method described by Escalante and Sastre [40] with minor modifications. Structures were developed on teflon® filters (Omnipore ™, Millipore Co., Badford, MA, USA), fixed and hybridized as described. 500 ng/mL of heat-denatured riboprobe were used for hybridization and colour reaction was stopped after 2 (streams) to 5 (culminants) hours. Pictures were taken (60X) with a camera (DFC420 Leica Microsystems, Wetzlar, Germany) attached to a stereo-microscope ($MZ9_5$ Leica Microsystems).

Both sense and antisense RNA probes were prepared by *in vitro* transcription and digoxigenin labelling of the complete *acaA* ORF (kindly supplied by P. Schaap) using a DIG RNA labelling kit (Roche Diagnostics Mannheim, Germany) according to the manufacturer's protocol.

Results

1. Structure of the *acaA* gene promoter region

The study of the structure of the promoter region was initiated by determining the transcription initiation site by primer extension using the rapid amplification of the 5′ cDNA ends (RACE) method. Several amplification products were obtained using RNA from growing cells and from structures isolated after 8 hours of development, which pointed to the existence of more than one transcription initiation site. Amplification products were cloned and their nucleotide sequence determined. The comparison of the nucleotide sequence of the RACE products (shown in Fig. 1) with that of the *D. discoideum* genome indicated that the *acaA* gene is transcribed from three different promoter regions distributed along the 4 kb long *lsm2-acaA* intergenic region. The location of the three alternative 5′-untranslated regions is schematically shown in Figure 1A. Each of the three promoters drove transcription of a different non-coding first exon of the mRNA (Fig. 1B). Splicing of the three different mRNAs joined these specific exons to a common second exon where the translation initiation codon is located (Fig. 1B). Therefore, the three different mRNAs code for the same AcA protein.

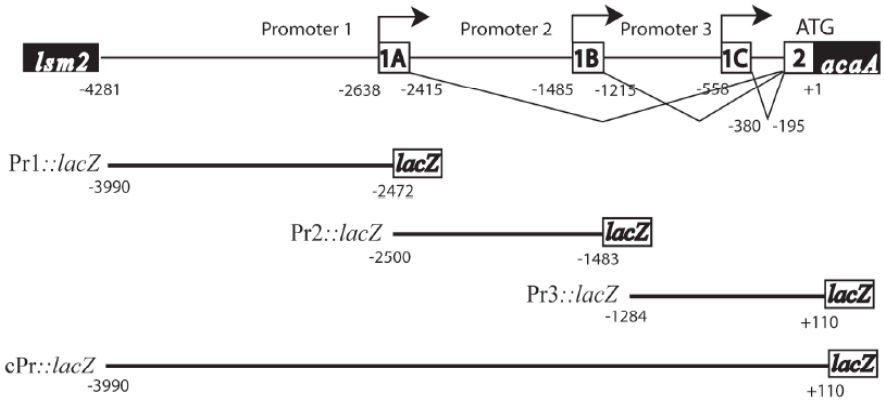
2. Cell-type specific activity of the *acaA* promoters

The existence of different promoters regulating the expression of one gene is often associated with complex patterns of expression. In many cases each of the alternative promoters is active in a particular cell type or at different stages of development or cell differentiation [35,36,41]. To ascertain if that could be the case for the *acaA* gene, the pattern of activity of each promoter during multi-cellular development was determined by histochemistry. A *lacZ* gene coding for short-lived β-galactosidase was used as reporter in these experiments. Each one of the three promoters, and the intergenic region including all three promoters, was cloned in the reporter vector, as schematically shown in Figure 1A. These constructs were transfected in *D. discoideum* cells and β-galactosidase activity determined at different developmental stages. Pools of transformed cells were used in these experiments to avoid possible differences due to clonal variations in plasmid copy number or integration sites.

The histological pattern of β-galactosidase activity expressed under control of promoter 1 is shown in Figure 2. This promoter showed maximal activity during aggregation. Cells that are at the streaming stage of aggregation showed high levels of β-galactosidase expression (Fig. 2A,B). The activity of this promoter could be still observed in cells located at the base of tight aggregates (Fig. 2C) but was not detectable at later developmental stages, such as slug structures (Fig. 2D) or in spores (Fig. 2E).

Promoter 2 showed expression in cells dispersed through mound structures (Fig. 3A), and, later on, in cells located in the basal region of tipped mounds (Fig. 3B). The cells where Promoter 2 was active localized to the posterior region in slugs (Fig. 3C), with a pattern compatible with anterior-like cell- or prespore- specific

A



B

Exon 1A:

ATTTTAAAAAATAAAGGCATTGGTCAGAAATATTATCATTTTTTATTTTTTTTTTTTTATTTTTAAAATAATTAACTTATCAATACAAATAATT
AGAAAATAACTAAATAATTAAAGTTTTTTTTTTTTGTTTTGTTTTTAGATACGAATTCTTGGATCTTTAAAAAATTAAATAACTAAAATATAAAA
AAAAAAAAAATAATTAATAAAAAAATAAAATTAAAAAAgtaagataaa

Exon 1B:

TTGTTATTTTTTTTTTATTTTTTTTTCTTTCTTTTTTTTTCTAATTGAATAACAAAATAAAAAAATTTATTTTTGTAAATACTGTCTTCAATAT
TCAATAAGTTGAAAATAGTCAATCAATTAATTGTTTGTTTAAAGTAGTATTTAGATTTAAAATAAAATAAATAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAATAACCTCACTTCATAAATATATCTTTGTAAATTAATCTTTCTTACTTTTATTTTTTATCTCAATTCTGTTATGgtaattatta

Exon 1C:

AGTAATATTATTACTATTATATAAAATAAAATAAAAATAAAAATAAAAATAAAATAAAAACTATTATCTAAAACAACTATATAAAATAATTTAT
TTTACTAGTAAAATTAATTTGTTGTACCATTAAAAATTTGTAATAAATAAATAAAAAAATATATTTATAATATGATTATTTATTgtacgcttca

Exon 2:

ttaatttaattatagTTAATTTTTTTTAAAAATATATTGTATTGATAGGAAAATAAAGTAGTAATAATAACAATAATAAATAATAAATAATAAT
AATAAAAAAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATAGTAATATTAAAATAGTAATAATAATAATAATCACAAAAAAAAAAAGTAAT
AATATTAAAAAATTATTAAAAA**ATGGCATCTAGCTCACCAATG**

Figure 1. Structure of the *acaA* gene promoter region. A. The 5′ untranslated regions of three different *acaA* mRNAs were identified by rapid amplification of cDNA ends and their nucleotide sequences compared to that of this region of the genome. The results are schematically shown in the upper panel where transcribed exon regions are indicated as boxes. Protein coding regions are indicated as black boxes and untranslated regions as open boxes. The upstream gene closest to *acaA*, *lsm2*, is located to the left of the scheme. The three promoter regions identified, Promoters 1, 2 and 3, are labeled in the upper part and transcription initiation sites indicated by arrows. Splicing events that generate the three different mRNAs are indicated with thin lines in the lower part of the scheme. The position of transcription initiation sites and exon limits is shown underneath and numbered in relation to the initiation codon. The lower part of the figure schematically shows the promoter fragments that were cloned in the Psa-ialphaGal reporter vector for functional analysis: Pr1::*lacZ*, Pr2::*lacZ*, Pr3::*lacZ* and the complete promoter, cPr::*lacZ*. Numbers relate to the ATG initiation codon. B. Nucleotide sequence of the three alternative first exons identified, Exons 1A, 1B and 1C, and the common second exon, Exon 2, as determined from the products of Rapid Amplification of cDNA End reactions. Exon sequences are shown in capital letters and adjacent intron sequences, obtained for the genome sequence, in small letters, showing the presence of conserved donor and acceptor splicing sites. The Exon 2 protein-coding region is shown in bold face characters.
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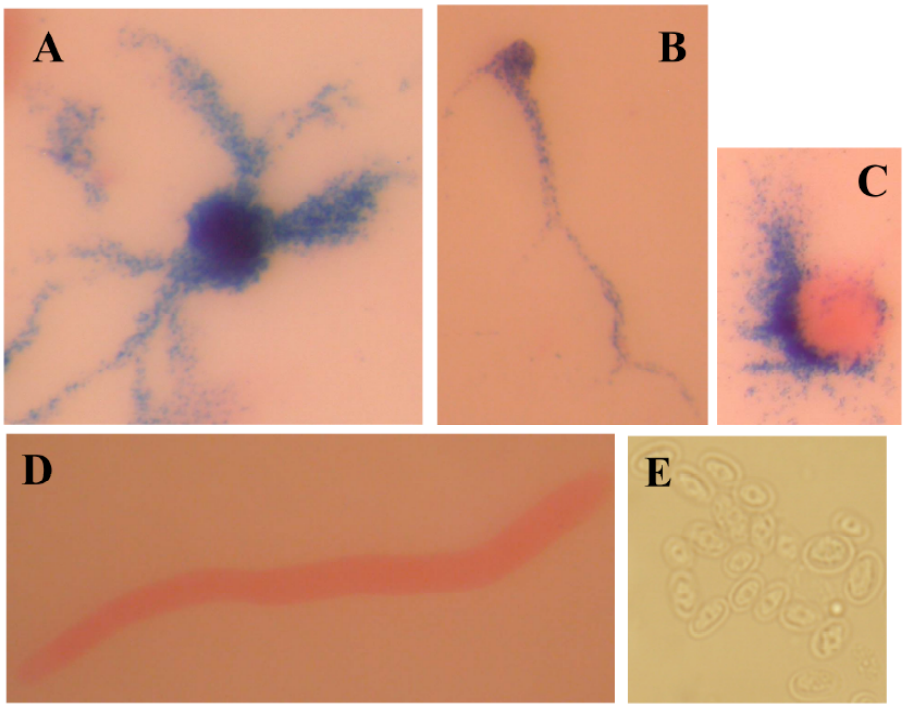

Figure 2. Activity of *acaA* Promoter 1 during *D. discoideum* development. *D. discoideum* AX4 cells were transformed with the reporter vector expressing the *lacZ* gene coding for short-lived β-galactosidase under the control of *acaA* Promoter 1. *lacZ* expression was detected by X-Gal hydrolysis and the structures were stained with eosine. Expression patterns obtained during cell aggregation (panels A and B), or at the early mound (C) and slug (D) stages of development and spores (E) are shown.
doi:10.1371/journal.pone.0013286.g002

expression, and to the mass of cells that migrate to the top of the structures during culmination (Fig. 3D, E). Expression was also observed in mature spores (Fig. 3F).

The more proximal promoter, Promoter 3, was active in a group of cells with the characteristics of prestalk tip-organiser cells and in prespore cells (Fig. 4). Expression was initially observed in cells dispersed throughout mound structures (Fig. 4A). At the first finger stage, Promoter 3 was active in cells at the tip of the structure (Fig. 4B). In slugs Promoter 3-expressing cells were found in the anterior part of the structure with a pattern similar to that of tip-organiser cells, and in cells scattered through the posterior part of the structure (Fig. 4C). During culmination, Promoter 3-expresssing cells were located in the tip of the structure and in the stalk that was formed from the tip, towards the substrate (Fig. 4D, E). This pattern of staining was maintained until the last stages of culmination (Fig. 4F). In addition, a weaker staining was observed in prespore cells (Fig. 4E.F) and in mature spores (Fig. 4G).

The combined activity of the three promoter regions was studied by cloning most of the intergenic region from the closest upstream gene (*lsm2*) to the *acaA* second exon in the same reporter vector used above. The complete promoter (cPr) showed a pattern of cell-type specific activity corresponding to the addition of the three individual promoters (Fig. 5). The complete promoter drove β-galactosidase expression at aggregation (Fig. 5A) and in cells evenly distributed in the mound (Fig. 5B) and tipped-mound structures (Fig. 5C). At the slug stage and during culmination maximal staining was observed in tip-organiser cells, located to the tip of the structures, and in the stalk (Fig. 5D-H). However, β-galactosidase activity was also observed in the prespore region of slug (Fig. 5D), mid-culminant (Fig. 5E,F) and late culminant (Fig. 5G, H) structures, and in spores (Fig. 5I). To ascertain these results the reporter vector containing the complete promoter was transfected in a different *D. discoideum* axenic strain, AX2 cells. The pattern of β-galactosidase expression observed was similar to that of AX4 cells. Although tip-organiser cells showed maximal expression, staining at aggregation and in the prespore region of slug (Fig. 5J) and culminant structures (Fig. 5K) was also observed.

3. Temporal pattern of promoter activity

The temporal pattern of activity was analyzed during development for each of the promoters by measuring β-galactosidase activity in cell extracts and the results are shown in figure 6A. In structures developed on Nitrocellulose filters, Promoter 1 was induced between 4 and 6 hours of development and remained active until 16 hours. Promoter 2 was induced between 6 and 10 hours of development and reached maximal activity between 14 and 16 hours to decrease thereafter. Promoter 3 was activated after 10 hours of development to remain active during the rest of the developmental process. The complete promoter was active from 6 hours of development although the main induction occurred between 10 and 14 hours, to reach a constant level of activity thereafter.

The activity of acaA promoters was compared to mRNA levels, estimated by quantitative RT-PCR. Oligonucleotides specific for

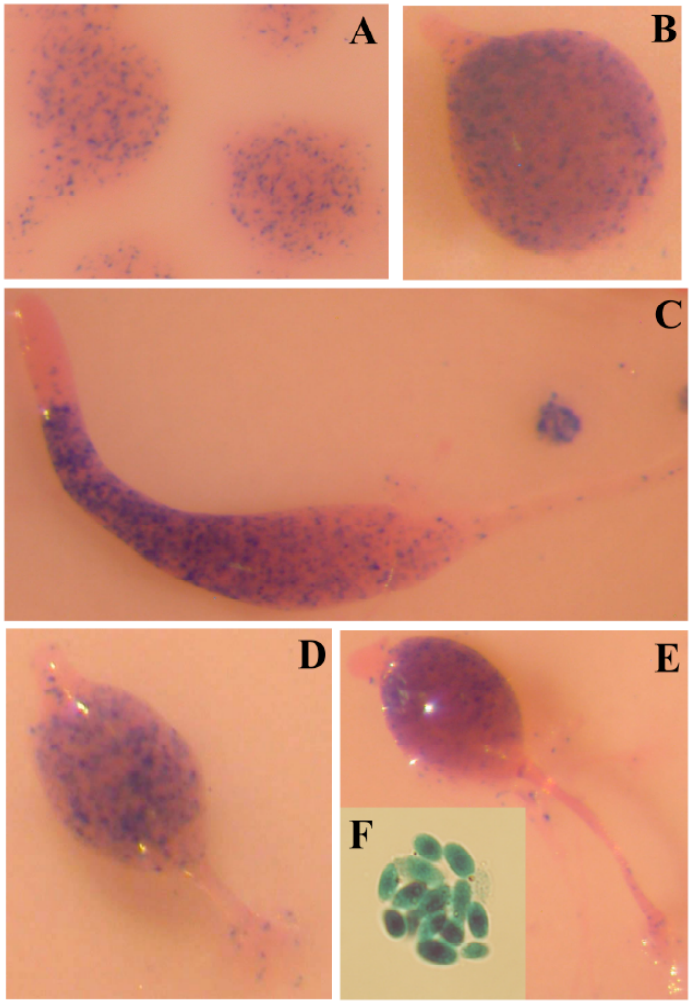

Figure 3. Activity of *acaA* Promoter 2 during *D. discoideum* development. AX4 *D. discoideum* cells were transformed with a reporter vector where the expression of a *lacZ* gene coding for short-lived β-galactosidase was under control of *acaA* Promoter 2. Transformed cells were allowed to enter multi-cellular development and *lacZ* expression determined by X-Gal hydrolysis at the early mound (panel A), tipped mound (B), slug (C), early culminant (D), late culminant (E) stages of development and spores (F). Structures were stained with eosine after X-Gal incubation.
doi:10.1371/journal.pone.0013286.g003

each of the three alternative first exons were designed and used in conjunction with a reverse oligonucleotide complementary to a region of the second exon that is common to all the mRNAs detected previously. This approach allowed specific detection of the mRNAs transcribed from promoters 1 (mRNA1), 2 (mRNA2) and 3 (mRNA3). Total acaA mRNA was detected using oligonucleotides that amplified a region of the common exon 2. Induction of mRNA1 and mRNA3 during development correlated well with the pattern of Promoter 1 and 3 activation, respectively. However, mRNA2 was induced before any increase in Promoter 2 activity could be detected, indicating that regulatory regions not present in this promoter region could regulate mRNA2 expression. mRNA1 and mRNA3 steady-state levels were lower than those of mRNA2. This difference was especially significant for mRNA3, which could be due to the very localized expression of this mRNA at the tip-organiser region. The levels of total mRNA expression (Exon2) correlated well with the added expression of the three specific mRNAs.

4. Analysis of *acaA* mRNA expression by in situ hybridization

The results obtained in the study of the promoter region were compared to the analysis of mRNA expression by in situ hybridization. Antisense and sense RNA probes specific for the *acaA* mRNA were generated and used for in situ hybridization of structures at different developmental stages (Fig. 7). Intense, scattered hybridization was first observed during early aggregation and in mounds (Fig. 7A, B). Later on, strong specific hybridization was observed at the prestalk and/or stalk region of tipped mounds (Fig. 7C), slug (Fig. 7D) and early culminants (Fig. 7F,G). We could

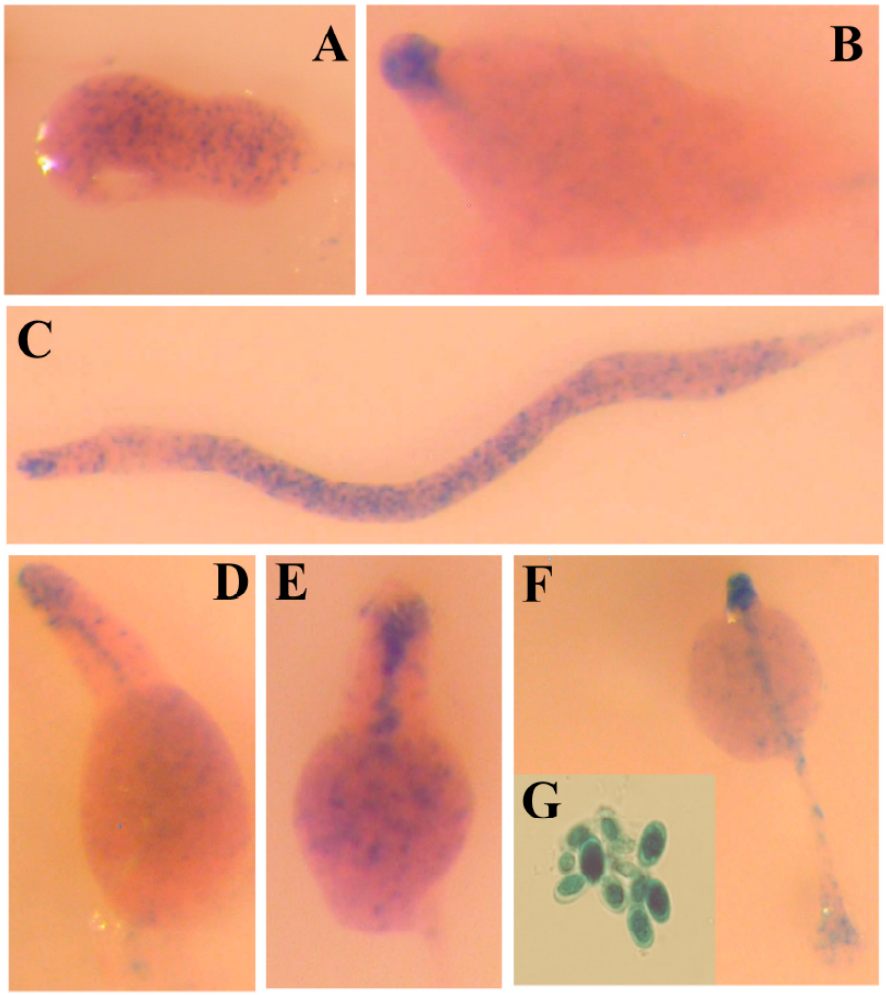

Figure 4. Activity of *acaA* Promoter 3 during *D. discoideum* development. *D. discoideum* AX4 cells were transformed with a vector where the reporter gene *lacZ*, coding for short-lived β-galactosidase, was under transcriptional control of *acaA* Promoter 3. *lacZ* expression was detected by X-Gal hydrolysis in the transformed strain at the early mound (A), finger (B), slug (C), early culminant (D), mid culminant (E), late culminant (F) stages of multi-cellular development and spores (G). Structures were stained with eosine after β-galactosidase detection.
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also observe moderate but specific hybridization in the prespore region of tipped mounds and slugs (Fig. 7C, D) and very week hybridization in the prespore region of early culminant structures (Fig. 7F, G). Incubation with a sense *acaA* RNA probe showed no specific hybridization (Fig. 7E,H). Both AX2 and AX4 developmental structures were analyzed by in situ hybridization and gave identical expression patterns.

Discussion

Primer extension analysis indicates that the *acaA* gene is transcribed from three alternative promoters, although the three mRNAs that are generated code for the same protein. Functional analysis indicates that the more distal, Promoter 1, is specifically active in aggregating cells. Promoter 2 is active in aggregates and in the posterior region of developmental structures, while the most proximal promoter, Promoter 3, is mainly active in prestalk cells. Promoter 3 activity is not detected in all the cells of the prestalk region but only in the more anterior ones, including the previously described tip-organiser region. The study of the complete promoter showed an integrated pattern of expression that included all three promoters and displayed a general profile more similar to Promoter 3.

The results obtained with the promoter analysis were supported by the in situ hybridization experiments and by the temporal patterns of expression observed for the three *acaA* transcripts. Quantitative RT-PCR experiments using oligonucleotides specific for each of the 5′-untranslated regions were used to specifically measure the level of expression of each one or the three mRNAs. The results obtained indicated that the three mRNAs are

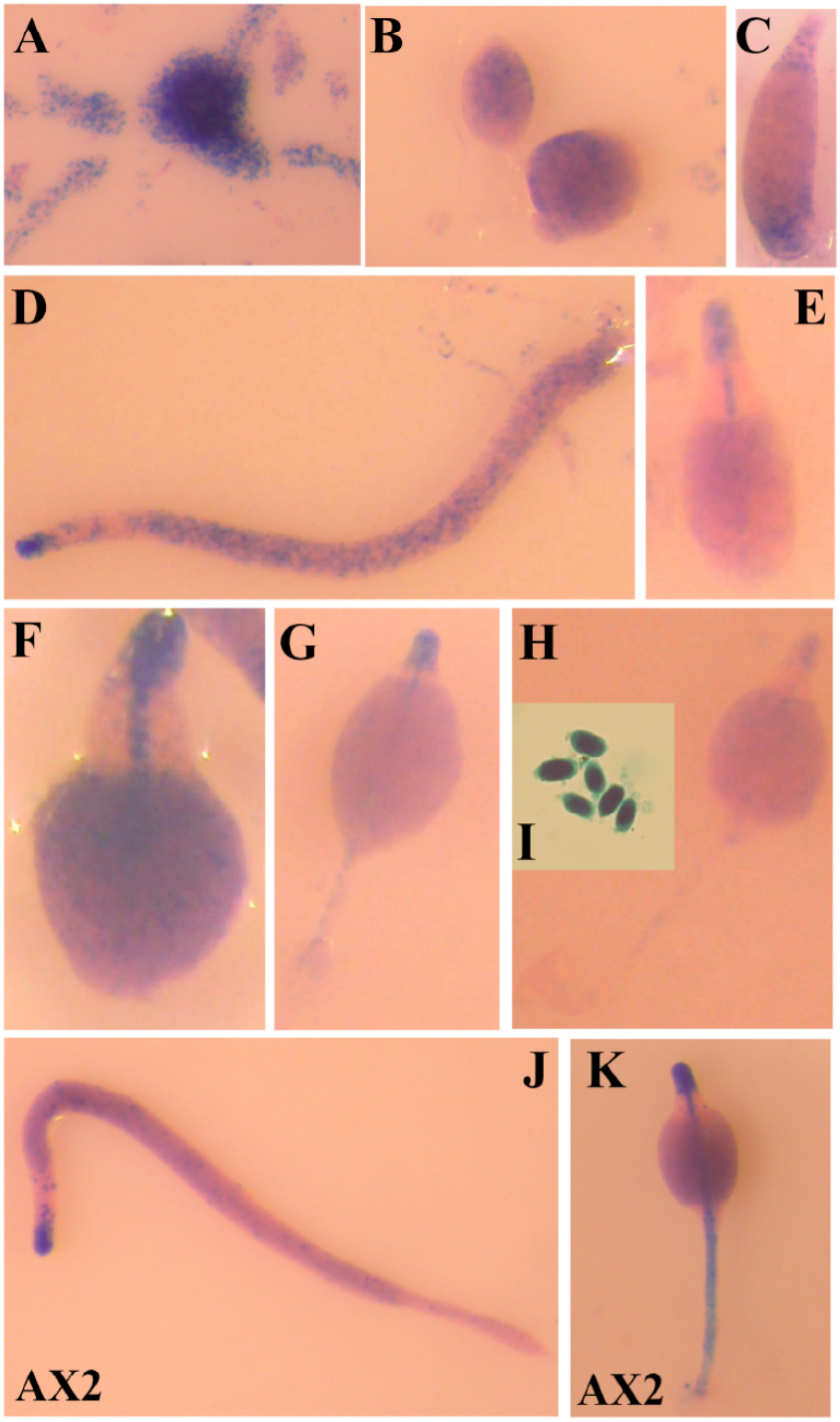

Figure 5. Activity of the complete *acaA* promoter during *D. discoideum* development. AX4 cells were transformed with a reporter vector where the complete *acaA* promoter region, covering most of the *lsm2/acaA* intergenic region, and including Promoters 1, 2 and 3, drives expression

of a *lacZ* gene coding for short-lived β-galactosidase. Transformed cells were starved and *lacZ* expression determined during aggregation (panel A), at the mound (B), finger (C), slug (D), early culminant (E, F), mid culminant (G) and late culminant (H) stages of development and in spores (I). The same vector was also transformed in *D. discoideum* AX2 cells and *lacZ* expression determined at the slug (J) and late culminant (K) stages of multi-cellular development. Structures were stained with eosine after the determination of β-galactosidase activity.
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expressed at different times during development. The mRNA transcribed from promoter 1 (mRNA1) was induced early during development to be repressed a few hours later, as also shown for promoter 1 activity. Induction and repression of the mRNA slightly preceded the observed variations in promoter activity. The temporal correlation between Promoter 3 activity and mRNA3 expression was fairly close with a marked increase between 10 and 14 hours of development to reach a plateau thereafter. More significant differences were found between the temporal pattern of Promoter 2 activity and mRNA2 expression, which is the more abundant and was induced six hours earlier than the observed increase in promoter activity. This result could be due to differences in the sensitivity of the detection method: Quantitative RT-PCR versus histochemistry. A second explanation could be the existence of interconnection between regulatory elements located in the different promoter regions analyzed. The experimental separation of the promoter in three regions according to the three transcription start points observed, which give the three alternative first exons, could be an oversimplification, even though they have proven to contain enough regulatory elements to regulate expression in different developmental structures. These promoter regions probably do not function independently and it could be expected that regulatory elements located in one promoter region affect the function of the others. In that case, β-galactosidase activity from the reporter constructs could differ from mRNA expression to some degree. That could be the case for Promoter 2 and mRNA2 where the early expression of the mRNA could be regulated by elements located outside of the Promoter 2 region analyzed. Interaction between regulatory elements located in different promoter regions could also explain the above-mentioned small differences observed between promoter 1 activity and mRNA1 expression.

Differences in the stability of *lacZ* and *acaA* mRNAs could also explain some of the discrepancies observed between the temporal patterns of promoter activity and mRNA levels during development. The three *acaA* mRNAs, and total mRNA, show sharper patterns of expression than those observed for promoter activity. mRNA steady-state levels increase and decrease before the corresponding levels of β-galactosidase activity. Given that a short-lived form of β-galactosidase has been used in these experiments, the differences could be explained if *acaA* mRNAs have a half-life shorter than *lacZ* mRNA. This difference seems to be especially significant at late developmental stages. Both Promoter 3 and the complete promoter are very active between 14 and 24 hours of development, as determined by β-galactosidase activity. However, *acaA* mRNA3 expression is maximal at 14 hours but decays markedly thereafter. The same decay is observed in total *acaA* mRNA steady-state levels, as also observed previously [29]. Since Promoter 3 activity is detected at the same developmental stage when mRNA3 starts to be expressed, these data could be explained by strong differences in *lacZ* and *acaA* mRNA stability.

Two studies on the *acaA* promoter region have been published previously. Verkerke-van Wijkt and collaborators [17] characterized an *acaA* promoter region isolated from a genomic clone. The comparison of the nucleotide sequence of this promoter and that of the genome indicates that the genomic clone contained an internal deletion that included part of Promoters 2 and 3 so that the promoter region studied was formed by a region of Promoter 2 (nucleotides −1838 to −1310) fused to the more proximal part of Promoter 3 (nucleotides −86 to 1). This promoter was active in cells at the mound stage and in tip-organiser cells, with a pattern of expression similar to the one shown for Promoter 3. These results could indicate the existence of a regulatory region activating gene expression at tip-organiser cells between nucleotides −86 and 1, that are also present in the Promoter 3 region analyzed in this article.

In addition Siol et al. [42] have characterized the promoter activity of a 773 bp fragment corresponding to the proximal region of Promoter 3 (nucleotides −739 to 34). This fragment activated transcription in aggregates and was dependent on the transcription factor CbfA. These authors also showed that CbfA was required for *acaA* expression during aggregation [43]. Since Promoter 1 is aggregation-specific, it will be of interest to determine if this promoter, that presents several possible binding sites for this transcription factor, is also dependent on CbfA for activation.

The expression of the *acaA* gene in prespore and prestalk cells in mound structures could be functionally relevant. An important difference between the three adenylyl cyclases is that their activity is regulated by different extracellular signals. Adenylyl cyclase A is homologous to G-protein coupled enzymes and its activity is regulated by G-protein coupled receptors through small G proteins [29,44]. cAMP receptors, such as Car1 that mediate the response to extracellular cAMP during aggregation belong to this family of proteins. During aggregation the presence of extracellular cAMP induces cAMP synthesis by AcA through Car1, establishing a feed-forward loop that is essential for cAMP signaling [45]. *acaA* mutant cells do not aggregate but the addition of extracellular cAMP or of 2′ deoxy cAMP, that does not activate protein kinase A, or the presence of wild type cells, enables aggregation of the mutant cells. Indeed, mutant *acaA* cells can complete development with the addition of extracellular cAMP suggesting that the main role of the encoded enzyme during development is extracellular cAMP production [46].

This regulatory capacity is unique to AcA, because the other two adenylyl cyclases are not activated by G proteins. AcG is an osmosensor molecule activated by high osmotic pressure of the extracellular media [22]. The mechanisms that activate AcB are presently unknown but it does not depend on G proteins [31]. Some domains of the AcB N-terminal region present similarity to proteins involved in two-component signaling pathways, indicating that its activity could be regulated by phosphorylation in response to extracellular signals.

As mentioned in the Introduction, despite detailed studies of single and multiple adenylyl cyclases mutants, there are still cAMP-dependent developmental processes where the respective contribution of these enzymes is not clear. Some of them are processes that occur between the acaA-dependent aggregation step [29] and culmination, that is dependent on acrA [31]. For example, the initiation of cell differentiation or the first morphogenetic processes of cell sorting and tip formation. *acrA* and *acgA* mutants can complete these developmental processes. *acrA* and *acgA* double mutants are defective in spore formation but still express significant levels of prestalk and prespore genes [32]. The contribution of *acaA* to these processes has been difficult to

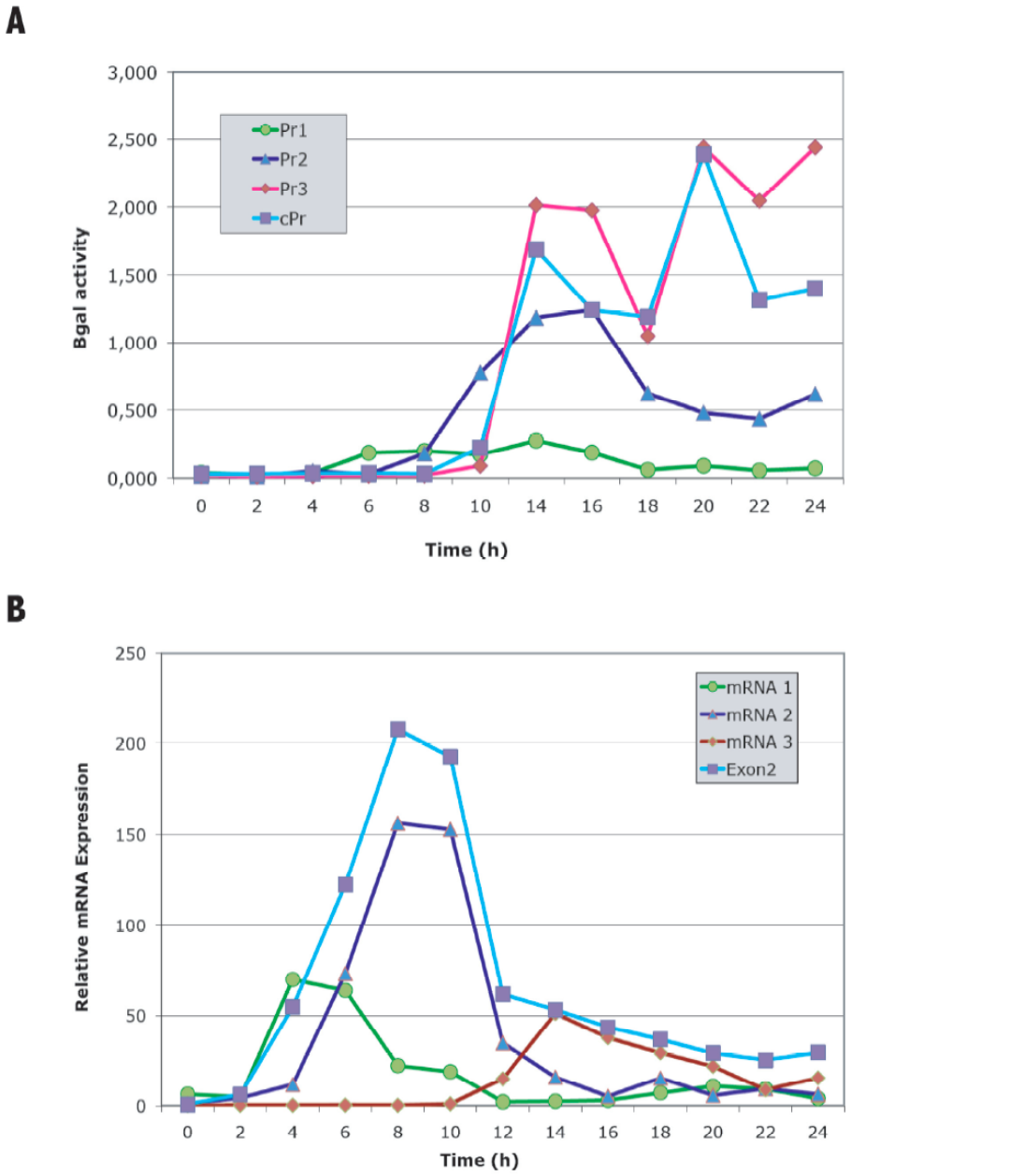

Figure 6. Analyses of acaA promoters activity and mRNAs expression during *D. discoideum* development. Panel A. *D. discoideum* AX4 cells were transformed with the reporter vectors where expression of a *lacZ* gene coding for short-lived β-galactosidase was under transcriptional control of *acaA* promotes 1, 2, 3 or the complete promoter. Transformed cells were allowed to develop on Nitrocellulose filters for the indicated hours (0–24). Collected samples were lysed and β-galactosidase activity determined using ONPG as substrate. A representative experiment where β-galactosidase activity was normalized by the amount of protein present in each sample is shown. (λ) Pr1::lacZ activity; (σ) Pr2::lacZ activity; (υ) Pr3::lacZ activity; (ν) cPr::lacZ activity. Panel B. RNA was extracted from growing cells (time 0) or from cells allowed to develop on Nitrocellulose filters for the indicated times (times 2–24). Expression of the different acaA mRNAs was analyzed by quantitative RT-PCR using oligonucleotides specific for the 5' region of the three mRNAs detected in the RACE analysis (mRNA-1 λ, -2 σ and -3 υ). Oligonucleotides that amplify an exon 2 region common to the three mRNAs were used to estimate total acaA mRNA expression (Exon 2 ν). Relative expression levels, derived form the cycle when amplification is first detected, are indicated. These levels have been multiplied by a factor of 5 for mRNA1 and of 100 for mRNA3, in relation to mRNA2 and Exon2 levels.
doi:10.1371/journal.pone.0013286.g006

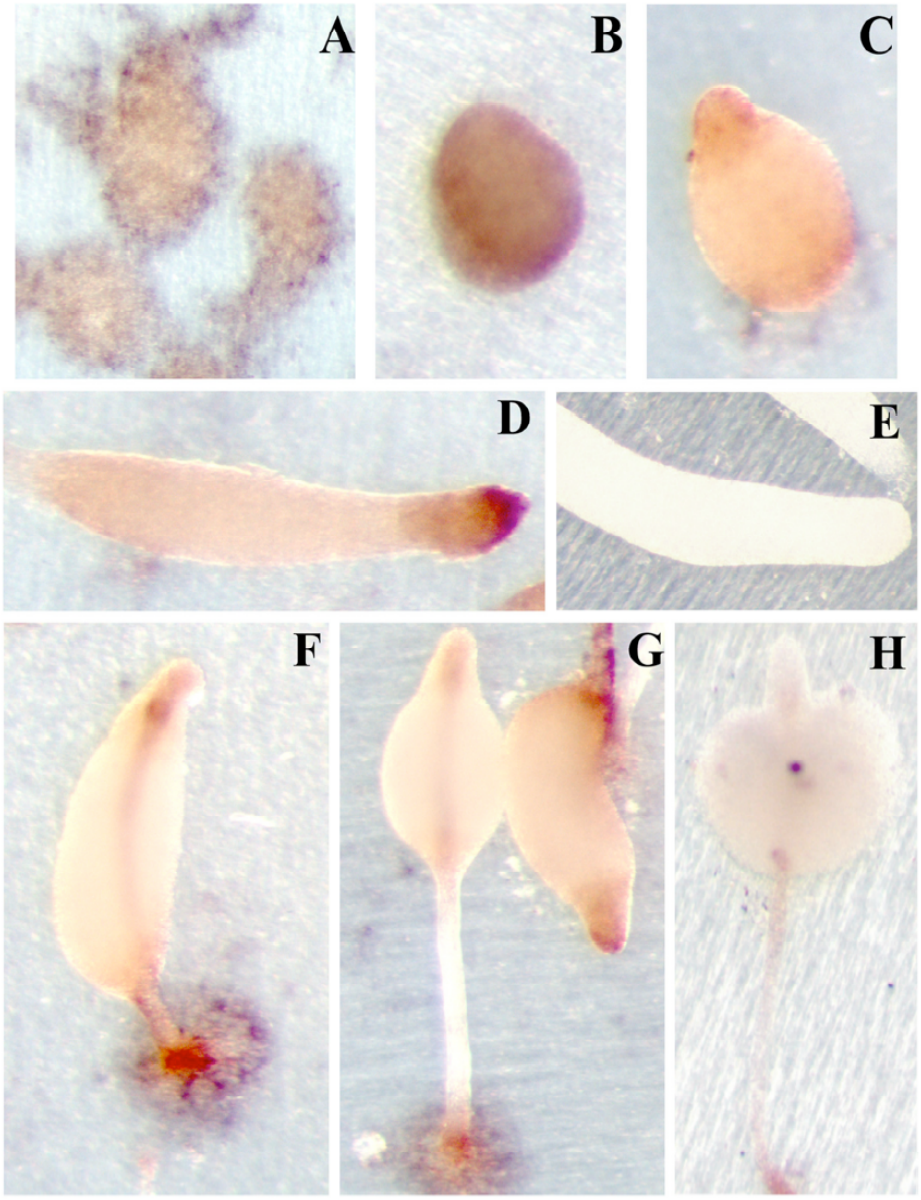

Figure 7. In situ hybridization analysis of *acaA* mRNA expression during *D. discoideum* development. *D. discoideum* AX4 cells were allowed to enter multi-cellular development on teflon® filters. Structures at the early aggregate (panel A), late aggregate (B), tipped mound (C), slug (D, E), early (F) and late culminant (G, H) stages of development were collected and *acaA* mRNA expression analyzed by in situ hybridization using an antisense RNA probe (A, B, C, D, F, G), or a sense RNA probe (E, H), as a control.
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determine because *acaA* mutants are not able to complete aggregation. However, the observation that *acaA* is expressed in the mound and in the tip-organiser region of finger and slug structures might indicate that AcA could be involved in cAMP signaling during these developmental stages. For example, it has been shown that cAMP waves continue to be formed from the upper part of the mound directing migration during cell sorting [47]. The expression of *acaA* at these structures and the regulatory capacity of the AcA enzyme would be in agreement with its implication in cAMP signaling during the formation of the tipped mound and slug structures. Similarly, AcA could be involved in the synthesis of the extracellular cAMP required for the first steps

of prespore differentiation. The decrease of *acaA* expression in the prespore region at later developmental stages and the data on the *acrA* and *acgA* mutants [32] would indicate that AcB and AcG could be more important for terminal spore differentiation.

The existence of a promoter region specifically active at the tip-organiser region can be of interest to better understand the regulation of culmination. Several elegant studies have contributed to establish a cAMP-dependent gene transcription cascade that regulates the initiation of culmination (reviewed in [3]). The process is initiated by activation of the STATa transcription factor by extracellular cAMP [17]. STATa induces expression of the CudA transcription factor [16] that consequently activates expression of tip-organiser genes such as expl7 [18]. Tip-organiser-specific expression of acaA could be the first step, necessary for extracellular cAMP synthesis that initiates this culmination-inducing cascade.

The presence of a distal promoter (Promoter 1) specifically active during aggregation might have interesting evolutionary implications. Schaap et al [48] have shown that Dictyostelids can be classified in four groups and that *D. discoideum* belongs to the only group that uses cAMP as a signaling molecule at aggregation [49]. In contrast, all Dictyostelids use cAMP as a signaling molecule for the last steps of multi-cellular development and cell differentiation [50]. Therefore, the regulation of aggregation by extracellular cAMP seems to be a recent adaptation of a group of Dictyostelids, including *D. discoideum*. Alvarez-Curto et al [50] found that this adaptation involved significant changes in the expression pattern of the *car1* gene, coding for the cAMP receptor involved in chemotaxis. Car1 is expressed at aggregation only in species that use cAMP as signaling molecule and this change was associated with the acquisition of a new distal promoter region specifically active during aggregation [41]. Another gene required for cAMP signaling, *pdsA* coding for a extracellular phosphodiesterase, also has an aggregation-specific distal promoter [51]. These data impelled Alvarez-Curto et al [50] to propose that the adaptation of Dictyostelids to the use of cAMP at aggregation had involved the acquisition of new transcriptional regulatory capacities through the incorporation of new promoter regions. In agreement with this hypothesis, *acaA* would be the third example of a gene involved in cAMP signaling that has an aggregation-specific distal promoter. The determination of the nucleotide sequence of the genome of more Dictyostelids will allow us to determine if this distal promoter is only present in species that use cAMP for aggregation.

In summary, the observation that *acaA* is transcribed from three different promoters, during aggregation and multicelular development indicates that this gene can be involved in more developmental processes than the previously known aggregation step. The identification of these promoter regions makes possible to approach the study of the mechanisms that regulate *acaA* expression at the different developmental stages. In addition, the study of the contribution of *acaA* to the different developmental processes can be approached by complementation studies using each of the specific promoters.

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Author Contributions

Conceived and designed the experiments: TS LS. Performed the experiments: MGC AG. Analyzed the data: MGC TS LS. Wrote the paper: LS.

References

1. Chisholm RL, Firtel RA (2004) Insights into morphogenesis from a simple developmental system. Nat Rev Mol Cell Biol 5: 531–541.
2. Annesley SJ, Fisher PR (2009) *Dictyostelium discoideum* - a model for many reasons. Mol Cell Biochem In press.
3. Williams JG (2006) Transcriptional regulation of Dictyostelium pattern formation. EMBO Rep 7: 694–698.
4. Konijn TM, van de Meene JGC, Bonner JT, Barkley DS (1967) The acrasin activity of adenosine-3′,5′-cyclic phosphate. Proc Natl Acad Sci USA 58: 1152–1154.
5. Jin T, Hereld D (2006) Moving towards understanding eukaryotic chemotaxis. Eur J Cell Biol 85: 905–913.
6. Siegert F, Weijer CJ (1995) Spiral and concentric waves organize multicellular Dictyostelium mounds. Curr Biol 5: 937–943.
7. Weijer CJ (2004) Dictyostelium morphogenesis. Curr Opin Genet Dev 14: 392–398.
8. Esch RK, Firtel RA (1991) cAMP and cell sorting control the spatial expression of a developmentally essential cell-type-specific ras gene in Dictyostelium. Genes Devel 5: 9–21.
9. Raper KB (1940) Pseudoplasmodium formation and organization in Dictyostelium discoideum. J Elisha Mitchell Sci Soc 56: 241–282.
10. Smith E, Williams K (1980) Evidence for tip control of the "slug/fruit" switch in slugs of Dictyostelium discoideum. J Embryol Exp Morphol 57: 233–240.
11. Saran S, Meima ME, Alvarez-Curto E, Weening KE, Rozen DE, et al. (2002) cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection. J Muscle Res Cell Motil 23: 793–802.
12. Dormann D, Vasiev B, Weijer CJ (2000) The control of chemotactic cell movement during Dictyostelium morphogenesis. Phil Trans R Soc Lond B 355: 983–991.
13. Barklis E, Lodish HF (1983) Regulation of Dictyostelium discoideum mRNAs specific for prespore or prestalk cells. Cell 32: 1139–1148.
14. Mehdy MC, Ratner D, Firtel RA (1983) Induction and modulation of cell-type specific gene expression in Dictyostelium. Cell 32: 763–771.
15. Wang B, Shaulsky G, Kuspa A (1999) Multiple developmental roles for CRAC, a cytosolic regulator of adenylyl cyclase. Dev Biol 208: 1–13.
16. Fukuzawa M, Williams JG (2000) Analysis of the promoter of the cudA gene reveals novel mechanisms of Dictyostelium cell type differentiation. Development 127: 2705–2713.
17. Verkerke-van Wijk I, Fukuzawa M, Devreotes PN, Schaap P (2001) Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression. Dev Biol 234: 151–160.
18. Wang HY, Williams JG (2010) Identification of a target for CudA, the transcription factor which directs formation of the Dictyostelium tip organiser. Int J Dev Biol 54: 161–165.
19. Hopper NA, Anjard C, Reymond CD, Williams JG (1993) Induction of terminal differentiation of Dictyostelium by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. Development 119: 147–154.
20. Berks M, Kay RR (1988) Cyclic AMP is an inhibitor of stalk cell differentiation in Dictyostelium discoideum. Dev Biol 126: 108–114.
21. Schilde C, Araki T, Williams H, Harwood A, Williams JG (2004) GSK3 is a multifunctional regulator of Dictyostelium development. Development 131: 4555–4565.
22. van Es S, Virdy KJ, Pitt GS, Meima M, Sands TW, et al. (1996) Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores. J Biol Chem 271: 23623–23625.
23. Berks M, Kay RR (1990) Combinatorial control of cell differentiation by cAMP and DIF-1 during development of Dictyostelium discoideum. Development 110: 977–984.
24. Anjard C, Chang WT, Gross J, Nellen W (1998) Production and activity of spore differentiation factors (SDFs) in Dictyostelium. Development 125: 4067–4075.
25. Schulkes C, Schaap P (1995) cAMP-dependent protein kinase activity is essential for preaggregative gene expression in Dictyostelium. FEBS Lett 368: 381–384.
26. Harwood AJ, Hopper NA, Simon MN, Driscoll DM, Veron M, et al. (1992) Culmination in Dictyostelium is regulated by the cAMP-dependent protein kinase. Cell 69: 615–624.
27. Mann SKO, Firtel RA (1993) cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during Dictyostelium development. Development 119: 135–146.
28. Kriebel PW, Parent CA (2004) Adenylyl cyclase expression and regulation during the differentiation of Dictyostelium discoideum. IUBMB Life 56: 541–546.
29. Pitt GS, Milona N, Borleis J, Lin KC, Reed RR, et al. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in Dictyostelium development. Cell 69: 305–315.

30. Kim HJ, Chang WT, Meima M, Gross JD, Schaap P (1998) A novel adenylyl cyclase detected in rapidly developing mutants of Dictyostelium. J Biol Chem 273: 30859–30862.
31. Soderbom F, Anjard C, Iranfar N, Fuller D, Loomis WF (1999) An adenylyl cyclase that functions during late development of Dictyostelium. Development 126: 5463–5471.
32. Alvarez-Curto E, Saran S, Meima M, Zobel J, Scott C, et al. (2007) cAMP production by adenylyl cyclase G induces prespore differentiation in Dictyostelium slugs. Development 134: 959–966.
33. Ritchie AV, van Es S, Fouquet C, Schaap P (2008) From drought sensing to developmental control: evolution of cyclic AMP signaling in social amoebas. Mol Biol Evol 25: 2109–2118.
34. Podgorski GJ, Franke J, Faure M, Kessin RH (1989) The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum utilizes alternate promotors and splicing for the synthesis of multiple mRNAs. Mol Cell Biol 9: 3938–3950.
35. Escalante R, Vicente JJ, Moreno N, Sastre L (2001) The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of Dictyostelium development. Dev Biol 235: 314–329.
36. Galardi-Castilla M, Pergolizzi B, Bloomfield G, Skelton J, Ivens A, et al. (2008) SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in Dictyostelium. Dev Biol 316: 260–274.
37. Pang KM, Lynes MA, Knecht DA (1999) Variables controlling the expression level of exogenous genes in Dictyostelium. Plasmid 41: 187–197.
38. Shaulsky G, Loomis WF (1993) Cell type regulation in response to expression of ricin-A in Dictyostelium. Dev Biol 160: 85–98.
39. Detterbeck S, Morandini P, Wetterauer B, Bachmair A, Fischer K, et al. (1994) The 'prespore-like cells' of Dictyostelium have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters. Development 120: 2847–2855.
40. Escalante R, Sastre L (2006) Investigating gene expression: In situ hybridization and reporter genes. In: Eichinger L, Rivero F, eds. Dictyostelium discoideum protocols. TotowaNJ: Humana Press. pp 230–247.
41. Louis JM, Saxe III CL, Kimmel AR (1993) Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during Dictyostelium development. Proc Natl Acad Sci USA 90: 5969–5973.
42. Siol O, Dingermann T, Winckler T (2006) The C-module DNA-binding factor mediates expression of the dictyostelium aggregation-specific adenylyl cyclase ACA. Eukaryot Cell 5: 658–664.
43. Winckler T, Iranfar N, Beck P, Jennes I, Siol O, et al. (2004) CbfA, the C-module DNA-binding factor, plays an essential role in the initiation of Dictyostelium discoideum development. Eukaryot Cell 3: 1349–1358.
44. Parent CA, Devreotes PN (1995) Isolation of inactive and G protein-resistant adenylyl cyclase mutants using random mutagenesis. J Biol Chem 270: 22693–22696.
45. Maeda M, Lu J, Shaulsky G, Miyazaki Y, Kuwayama H, et al. (2004) Periodic signaling controlled by and oscillatory circuit that includes protein kinases ERK2 and PKA. Science 304: 875–878.
46. Pitt GS, Brandt R, Lin KC, Devreotes PN, Schaap P (1993) Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in Dictyostelium cells lacking the aggregation adenylyl cyclase (ACA). Genes Devel 7: 2172–2180.
47. Siegert F, Weijer CJ (1992) Three-dimensional scroll waves organize Dictyostelium slugs. Proc Natl Acad Sci USA 89: 6433–6437.
48. Schaap P, Winckler T, Nelson M, Alvarez-Curto E, Elgie B, et al. (2006) Molecular phylogeny and evolution of morphology in the social amoebas. Science 314: 661–663.
49. Schaap P (2007) Evolution of size and pattern in the social amoebas. Bioessays 29: 635–644.
50. Alvarez_Curto E, Rozen D, Ritchie A, Fouquet C, Baldauf SL, et al. (2005) Evolutionary origin of cAMP-based chemoattraction in the social amoebae. Proc Natl Acad Sci U S A 102: 6385–6390.
51. Faure M, Franke J, Hall AL, Podgorski GJ, Kessin RH (1990) The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains 3 promoters specific for growth, aggregation, and late development. Mol Cell Biol 10: 1921–1930.

Capítulo 3: *The transcription factor SrfB regulates Adenylil cyclase A (acaA) expression during Dictyostelium discoideum development.*

El factor de transcripción SrfB de *Dictyostelium discoideum* es una proteína homóloga al factor SRF ("Serum Response Factor") de animales. En *D. discoideum* participa en varias etapas del proceso de desarrollo que desemboca en la formación del cuerpo fructífero. En este artículo hemos intentado vislumbrar el posible papel que srfB desempeña en la regulación del inicio de la culminación. Los resultados obtenidos indican que srfB podría localizarse antes de la cascada de señalización que inicia la culminación. Dicha cascada comienza con la formación de altas concentraciones extracelulares de AMPc, por parte de las encimas Adenilato ciclasas que activan, a su vez, la translocación al núcleo del factor de transcripción StatA y la activación de la expresión del factor CudA y de sus genes diana. De las tres Adenilato ciclasas existentes estudiamos AcaA, encima que se encuentra implicada en la fase de agregación y que se expresa al principio de la culminación. Los estudios realizados en cuanto a la expresión del gen de la Adenilato ciclasa A (*acaA*) en la cepa mutante para *srfB* indican que srfB podría participar en la regulación de la expresión de dicho gen. La expresión de *acaA* en la región organizadora del *"tip"*, dirigida por el Promotor 3, presenta una menor intensidad y además la región está menos definida, las células se encuentran más dispersas en el mutante de *srfB*. La expresión de *acaA* en la región pre-espora, bajo la dirección del Promotor 2, es significativamente menor en los mutantes para *srfB*. Estos datos indican que algunos de los defectos que presenta el mutante para *srfB* durante el desarrollo, como es el retraso en la culminación, podrían ser debidos a una expresión defectuosa de la Adenilato ciclasa (*acaA*) que, como consecuencia, produce cambios en la señalización intercelular mediada por AMPc. Cuando se llevaron a cabo experimentos de mezclas entre células *"wild type"* y mutantes de *srfB* se obtuvieron datos indicativos de que la expresión de *acaA* en la región pre-espora puede estar regulada a través de mecanismos de interconexión celular dependientes de srfB.

The transcription factor SrfB regulates adenylyl cyclase A (*acaA*) expression during *Dictyostelium discoideum* development.

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SUMMARY

The *Dictyostelium discoideum* SrfB transcription factor, homologous to animal SRF (Serum Response Factor), participates in several aspects of the developmental process that conduces to fruiting body formation. In particular, the contribution of SrfB to the regulation of culmination initiation has been studied in this article. The results obtained indicate that SrfB could be placed upstream of the well-characterized cAMP/STATa/CudA regulatory pathway. Furthermore, analysis of the expression of the *acaA* gene, coding for adenylyl cyclase A, in *srfB*-mutants indicated that SrfB participates in the regulation of the expression of this gene. Expression of *acaA* at the tip-organizer region, driven by the alternative promoter 3, is weaker and follows a more disperse pattern in *srfB* mutants. In addition, *acaA* expression in the prespore region, under Promoter 2 control, is significantly decreased in *srfB* mutants. These data indicate that some of the developmental defects observed in *srfB*-mutant strains, such as delayed culmination, could be due to alterations in *acaA* expression that results in changes in cAMP inter-cellular signalling. The study of Wild-type/*srfB*-mutant chimeric structures also indicated that *acaA* expression in the prespore region could be regulated through SrfB-dependent intercellular signalling mechanisms.

INTRODUCTION

D. discoideum fruiting body formation is one of the more primitive examples of multicellular development but, even so, is a complex process that is tightly regulated (Williams, 2006). The process is initiated when free-living amoeba get under starvation conditions. In this case, cells initiate a differentiation program and up to 10^5 aggregate together to form cell mounds that develop into fruiting bodies. Cell aggregation is mediated by cAMP in *D. discoideum* (Konijn et al., 1967). Cells in aggregation fields are able to move towards increasing cAMP concentrations and to secret cAMP, so that the signal gets amplified (Jin and Hereld, 2006). Upon aggregation, mounds are formed where cells differentiate in two main alternative pathways: prestalk or prespore cells. Prestalk cells associate together and move to the upper part of the mound, where they emerge as a tip, while prespore cells remain in the lower part of the mound (Esch and Firtel, 1991). The tip region acts as an organizing centre during later development (Raper, 1940; Smith and Williams, 1980). Culmination takes place when prestalk cells, located in the tip, migrate towards the substrate through the mass of prespore cells, elongate and synthesize a cellulose outer layer, as a consequence, prespore cells are raised from the substrate to form a sorus, and complete the differentiation process into spores.

D. discoideum development is coordinated by transcription factors specifically active in different cell types or developmental stages. Among them are two transcription factors, SrfA and SrfB, homologous to the Serum Response Factor (SRF) that plays an important role in the regulation of cellular differentiation and development in animals. On the one hand, *srfA* is involved in spore differentiation and slug migration (Escalante, 2003) (Escalante et al., 2004). On the other hand, *srfB*, described few years ago (Galardi-Castilla M, 2008), is expressed during proliferation and multicellular development under the control of three different promoter regions, which show different cell-type specific patterns of expression. *SrfB* mutant cells showed several defects both during growth and development, consistent with the complexity of the promoter. *srfB*$^-$ cells showed alterations in cytokinesis and pynocitosis during proliferation and impaired migration and adhesion to the substrate during early development. These defects could be related with an impaired cytoskeleton function. Mutant cells also displayed an altered starvation response, faster than the wild type one. Aggregates are formed on nitrocellulose or agar about 2 h earlier by *srfB*$^-$ than by wild-type cells. Besides, *srfB* mutant cells suffer a misregulation of several genes implicated in proliferation/development transition. During development, chemotaxis to cAMP, the main process that directs cell aggregation, and acquisition of EDTA-stable cell adhesion, are impaired. However, the acquisition of EDTA-labile adhesion was increased in the mutant which might explain the rapid formation of small clumps, in the absence of chemotaxis, on nitrocellulose filters or agar. *srfB*-mutant strains also

showed a delay in culmination and stay as migratory structures (slugs) a longer time than wild-type structures, indicative of a defect in the coordination of the developmental process.

Several signalling molecules coordinate *D. discoideum* development but, among them, cAMP plays a central role (Chisholm and Firtel, 2004; Saran S, 2002). Extracellular cAMP first mediates aggregation. Later on, cAMP secreted from the tip is required for prestalk and prespore cells sorting in the mound (Dormann et al., 2000). Extracellular cAMP at a high, constant level is required for in vitro prespore and prestalk differentiation, inducing or repressing the expression of cell-type specific genes. The decision of initiating culmination is also dependent on extracellular cAMP, that activates the STATa transcription factor at the tip organizer region, in the anterior region of the slug, initiating a regulatory cascade that proceeds through activation of the CudA transcription factor (Fukuzawa and Williams, 2000; Verkerke-van Wijk et al., 2001) (Williams, 2006) and the tip-organizer-specific expression of their target genes, such as expl17 (Wang HY, 2010). Also, cAMP is necessary for activation of protein kinase A at the beginning of development (Schulkes and Schaap, 1995) and for spore differentiation (Harwood et al., 1992; Mann and Firtel, 1993).

The extensive use of cAMP as signalling molecule requires a tight control of its synthesis and degradation. Synthesis is catalyzed by three different adenylyl cyclases, encoded by the genes *acaA, acrA y acgA*. Degradation is carried out by extracellular phosphodiesterases, such as PDE, and intracellular ones, such as RegA or PdeE. The expression of these enzymes is regulated by extracellular signals during development stablishing, thereby, a precise control of cAMP levels (Kriebel PW, 2004).

Adenylyl cyclase A is a development-specific enzyme whose synthesis is induced during aggregation (Kriebel PW, 2004). The study of the promoter region of *acaA* gene indicated that is transcribed from three alternative promoters. The more distal, Promoter 1, is specifically active in aggregating cells. The promoter 2 is active in aggregates and in the posterior region of developmental structures, while the most proximal promoter, Promoter 3, is mainly active in prestalk cells, but in finger and slug structures is particularly active in the previously described tip-organizer region. The study of the complete promoter show an integrated pattern of expression that include all three promoters and display a general profile more similar to Promoter 3 (Galardi-Castilla M, 2010). The observation that *acaA* is expressed in the tip-organizer region might indicate the implication of the encoded enzyme in the culmination process.

The expression of *srfB* in the tip-organizer region and the defects observed for the mutant strain in culmination indicated that this gene takes part of the regulatory circuit that regulates culmination and that include *acaA*, *statA*, *cudA* and CudA-regulated genes such as *expl17*, as mentioned above. The results shown in the article indicate that SrfB might be upstream of the

STATa/CudA pathway and that participates in the regulation of *acaA* expression at the tip-organizer and prespore regions in slug and culminant structures.

MATERIALS AND METHODS

Strains, Cell culture, transformation and development

D. discoideum cells were cultured axenically in HL5 media. Transformation by electroporation was performed as described (Pang et al., 1999). Transformed cells were selected by treatment with blasticidin (Adachi et al., 1994) or neomycin (G418). Filter development was induced by spreading 1-2 x10^7 (0.6-1.2 x 10^6 cells/cm^2) on Nitrocellulose filters (Millipore Co., Bradford, MA, USA) (Shaulsky and Loomis, 1993). *dstA*$^-$ (Dd-STATa null mutant) and *cudA*$^-$ (CudA null mutant) strains were supplied by Dictybase stock centre (http://dictybase.org/db/cgi-bin/dictyBase/SC/strainlist.pl).

Construction of reporter vectors

Reporter vectors carrying *lacZ* under the control of *acaA* promoters (Promoter 1, Promoter 2, Promoter 3 and Complete Promoter) were constructed previously in our laboratory and were used for transforming wild type cells and *srfB* mutant cells (Galardi-Castilla M, 2010). *EcmB*-i-alpha-gal and *cudA*-i-alpha-gal vectors were supplied by the Dictybase stock centre. A previously described *acaA* promoter reporter vector (23) was supplied by Dr. Pauline Shaap, University of Dundee, UK.

Hystochemical determination of β-galactosidase activity.

Cells transformed with different reporter vectors were allowed to develop on Nitrocellulose filters for the periods of time indicated in each experiment. Structures were fixed, permeabilized and the β-galactosidase activity detected by X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopiranoside) hydrolysis as a previously described (Escalante and Sastre, 2006).

Determination of mRNA levels by quantitative RT-PCR

RNA was isolated from 2x10^7 cells, either at growth or after development on Nitrocellulose filters for the times indicated in each experiment, using the TRI reagent® (Sigma-Aldrich, Inc, St Louis, MO, USA) according to the manufacture's instructions. RNA was purified using the Quiagen Rneasy® Mini kit (Ambion, Inc., Austin, TX, USA). CDNA's were synthesized from 2 μg of total RNA using random primers (Promega Co, Madison, WI, USA). cDNA's were used as substrates for quantitative real-time PCR reactions using gene specific oligonucleotides. Promoter 1 was amplified using the oligonucleotides 5'-

GGAGATCTACCACCACCATTTCCATCATG-3', complementary to nucleotides 90 to 110 of the gene, encoded in Exón2 and 5'-GGTCTAGAGTTTTTAGATACGAATTCTTGG-3'. Promoter 2 was amplified also using the oligonucleotides 5'-GGAGATCTACCACCACCATTTCCATCATG-3' and 5'-GGTCTAGACCTCACTTCATAAATATATCTT-3'. Promoter 3 was amplified using oligonucleotides 5'-GGAGATCTACCACCACCATTTCCATCATG-3' and 5'-CTAGTAAAAATTAATTTGTTGTACC. The common exon 2 was amplified using oligonucleotides 5'-GGAGATCTACCACCACCATTTCCATCATG-3' and 5'-GGCATCTAGCTCACCAATG-3'. A region of the large mitochondrial ribosomal RNA was amplified as a loading control using the oligonucleotides 5'- CACTTTAATGGGTGAACACC-3' and 5'- GGGTAGTTTGACTGGGGCGG-3. The Step one plus real time PCR system® (Life technologies Co - Applied Biosystems, Carlsbad, CA, USA) was used in these experiments. PCR products were labelled with Sybr-green using the Power Sybr®-Green PCR Master Mix (Applied Biosystems) reaction mix following the manufacturer's instructions. The final volume of the reaction was of 20 μl, using a 0,2 μM concentration of each primer. PCR conditions were as follows: 95ºC, 10 m (95ºC, 15 s; 45ºC, 30 s; 62º 1 m) x 30-40 cycles.

RESULTS

1. Initiation of culmination in *srfB*-mutant strains

The beginning of the culmination in *D. discoideum* is determined by an increased cAMP extracellular concentration. This extracellular signal triggers, in the top cells of the structure, a signalling transduction pathway that starts the culmination process. STATa translocation to the nucleus of tip cells activates the expression of the CudA transcription factor that activates the expression of other effector genes (schematically shown in the right panel of Fig 1B). In order to determine if srfB is involved in the StatA signalling pathway, *srfB*$^-$ mutant cells were transfected with reporter vectors that drive *lacZ* expression under the control of the *cudA* promoters and that of *ecmB*, a tip-organizer region and stalk marker. Expression of the *cudA* reporter vector was observed in prespore and spore cells in developmental structures both in wild-type and *srfB*$^-$ mutant strains. However, *cudA* promoter was active in the tip-organizer region in wild type, but not in *srfB*-mutant slugs (Fig 1A). However, *ecmB* was expressed in the tip-organizer region and in scattered cells in the posterior region of both wild-type as *srfB*-mutant slugs. Moreover, *ecmB* is expressed in the stalk, lower and upper cup regions in both strains (Fig1A).

The interaction of SrfB with this signalling pathway was further characterized by studying the activity of the *srfB* gene promoter in *cudA*- and *statA*- mutant strains (Fig 1B). *SrfB* promoter was active in the tip-organizer region of the *cudA*$^-$ mutant strain, as previously reported for wild-type, AX4 cells (Fig1B, left panel). The *srfB* promoter was also active in the tip region of *statA*$^-$ mutant finger and midculminant structures (Fig1B, central panels). *srfB*$^-$ expressing cells were not concentrated in the tip-organizer regions probably because *statA*-mutant fingers are defective in culmination and do not form a properly defined tip-organizer. Together, these results indicate that the expression of *srfB* is independent of the CudA/StatA pathway (Fig1B, right panel) and can be located upstream of the STATa and CudA proteins.

2. Cell-type specific activity of the *acaA* promoters in *srfB*-mutant structures

Extracellular cAMP initiates the previously studied signalling pathway that induces culmination. The expression of *acaA* in the tip-organizer cells indicates that this enzyme is probably responsible for the synthesis of the cAMP that initiates this process. Therefore, the expression of the *acaA* gene in the *srfB*-mutant strains was studied. As mentioned in the Introduction, *acaA* is expressed in different regions of developing structures from three alternative promoters. The pattern of activity of each promoter in *srfB* mutant and wild-type cells was determined by hystochemistry using reporter vectors. A *lacZ* gene coding for short-lived β-galactosidase was used as reporter in these experiments. The reporter vectors with each promoter were transfected in *D. discoideum* wild-type and *srfB* mutant cells and β-galactosidase

activity determined at different developmental stages. In wild type cells, Promoter 1 showed maximal activity during aggregation, including streams and mound structures, but was not detectable at later developmental stages (Galardi-Castilla M, 2010). Wild-type and *srfB*$^{-}$ mutant cells transfected with promoter 1 driving *lacZ* expression showed the same pattern of expression (Fig2A).

The promoter 2 showed activity in cells dispersed through mound structures, and, later on, in cells located in the lower region of tipped mounds in wild type cells. In slugs, promoter 2 was active in cells localized in the posterior region, with a pattern compatible with anterior-like cells or prespore specific expression. Later on, the activity was observed in the sorus of culminated structures (Fig2B, upper panels). The expression of promoter 2 in *srfB*-mutant structures presented a similar pattern but the level of expression was much lower than in wild type one (Fig2B, lower panels). As srfB was not expressed in the prespore region, these results could be explained by the existence of intercellular signalling pathways originated in prestalk cells, where *srfB* is expressed, and that regulate *acaA* expression in prespore cells.

The more proximal *acaA* promoter, Promoter 3, was initially active in wild type cells dispersed throughout mound structures. In slugs, labelled cells were found in the anterior part of the structure with pattern similar to that of tip-organizer cells (Fig3A, upper panels) and in cells scattered through the posterior part of the structure. During culmination, expressing cells were located in the tip of the structures and in the forming stalk region (Fig3A, upper panel). In *srfB* mutant structures, the distribution of cells, where Promoter 3 was active, presented subtle differences with respect to wild type ones. The expression in the anterior part of the slug was weaker and the cells were not organized in an as well-defined tip-organizer region. In adition, the activity of the promoter in the stalk region was also weaker in the *srfB*-mutant structures (Fig3A, lower panels). Previously, Verkerke-van et al (Verkerke-van Wijk et al., 2001) had described the activity of a region of the *acaA* gene promoter. This promoter was formed by a region of Promoter 2 fused to the proximal region of the Promoter 3 and was specifically active in tip-organizer cells. This promoter was transfected in Ax4 and *srfB*-mutant strains. The pattern of expression in Ax4 was the same that was described by Verkerke-van et al (Fig3B, upper panels). However, the activity of this *acaA* gene promoter was not detected in slug and mid culminant structures from the *srfB*-mutant strain (Fig3B, lower panels).

The expression of the *acaA* Complete Promoter was also studied in Ax4 and *srfB*-mutant strains (Fig4). In both strains the complete promoter showed a pattern of cell-type specific activity corresponding to the addition of the three individual promoters. Activity was located in cells evenly distributed in mound and tipped-mound structures. The activity in slugs and culminant structures was found in tip-organizer cells, in the stalk and also observed in the prespore region. Activity of the complete promoter was also significantly lower in the prespore region of *srfB*-mutant structures than in AX4 structures. Similarly, activity in the tip region was

weaker and expressing cells were organized in a broader area that in wild-type structures. Activity in the growing stalk region was also weaker in mutant structures (Fig4).

Together, these experiments could indicate that *srfB* regulate ACA in prespore region, but since *srfB* is mainly expressed in the prestalk region the existence of a signalling pathway between prestalk and prespore cells could be defective in the *srfB* mutant. Chimeric structures were generated to study this possibility. Mutant cells, wild type cells, and wild-type and mutant cells transfected with reporter vectors where Promoter 2 drive *lacZ* expression were mixed in a 4:1 proportion and allowed to develop as slug structures. Promoter 2, as mentioned before, showed activity in the prespore region of slug structures in wild type cells (Fig5, AX4/pr2::*lacZ;* AX4//AX4/pr2::*lacZ*). Whereas that, *srfB* mutant cells also presented a weak level of activity in the prespore region (Fig 5, *srfB*$^-$/pr2::*lacZ; srfB*$^-$//*srfB*$^-$/pr2::*lacZ*). When wild type cells were mixed with mutant-Promoter2 transfected cells the expression of *lacZ* was localized in scattered cells of the prespore region, at a very low level (Fig5, AX4//*srfB*$^-$/pr2::*lacZ*). Unexpectedly, when we mixed *srfB* mutant cells and wild-type-Promoter2 transfected cells the expression in the posterior region was also very low (Fig5, *srfB*$^-$//AX4/pr2::*lacZ*). Hence, the analysis of the chimera indicates that the absence of *srfB* could have an inhibitor effect on *acaA* expression at the posterior region of slug structures.

3. Expression of *acaA* transcripts in wild-type and *srfB* mutant cells.

The expression of the three transcripts originated from each of the alternative promoters during development was analyzed by quantitative RT-PCR experiments. Oligonucleotides specific for each of the three alternative first exons were designed and used in conjunction with a reverse oligonucleotide complementary to a region of the second exon that is common to all the mRNAs, as previously described (8). Total *acaA* mRNA was detected using a oligonucleotides that amplified a region of the common exon2 (Galardi-Castilla M, 2010). As previously was described, mRNA1, transcribed from Promoter 1, was induced early during development in wild type structures. However, *srfB* mutant cells presented higher expression during vegetative growth that was induced by 2 hours and maintained up to 8 hours of development (Fig6A).

Expression of mRNA2, transcribed from promoter 2, was induced by 6-8 hours of development in AX4 structures. Expression in *srfB* mutant structures was lower than in wild type ones and decreased earlier at 12 and 14 hours of development (Fig6B). That correlates with the pattern of activation of Promoter 2 showed in Fig 2B. mRNA3 expression, regulated from Promoter 3, was similar in both strains since no significant differences were observed (Fig6C). Total *acaA* mRNA, as detected by the analysis of Exon 2 expression, was induced during development, reaching a peak of expression at 8 hours to decrease thereafter in wild-type structures, as previously described (8). Total *acaA* mRNA expression in *srfB* mutant cells and

structures showed a pattern similar to the wild-type one, except that expression in growing cells (time 0) was higher, the induction was a little earlier reaching maximal levels at 6 hours of development and that the decrease in expression at later developmental stages was more pronounced (Fig6D). The differences observed in Total *acaA* mRNA expression are in agreement with the higher mRNA1 expression observed in mutant cells at the first hours of development and with the lower expression observed for mRNA2 from 8 hours of development.

DISCUSSION

The contribution of the SrfB transcription factor to the induction of culmination of *D. discoideum* fruiting body structures has been studied in this article. Culmination is induced by extracellular cAMP signals that initiate an intracellular signal transduction pathway that sequentially activates the transcription factor STATa, CudA expression and the transcription of its target genes (Williams, 2006). The results obtained in this article indicate that SrfB either works upstream of this pathway or previously to its activation. In support of this hypothesis, *cudA* is not expressed in the tip-organizer region of *srfB*$^-$ structures. In addition, *srfB* is properly expressed at the tip-organizer region of *statA* and *cudA* mutant structures. However, a classical tip-organizer gene, *ecmB* is properly expressed in *srfB* mutants, indicating that tip-organizer cells are specified in this mutant but do not proceed through the culmination initiation process. *EcmB* is also expressed in the tip-organizer region of *cudA* mutant structures (Fukuzawa et al., 1997).

The signalling molecule that initiates culmination, cAMP, is synthesized by adenylyl cyclases. There are three adenylyl cyclases in *D. discoideum*, encoded by the genes *acaA*, *acgA* and *acrA* (Kriebel PW, 2004). Among them, *acaA* is the cyclase expressed at the tip-organizer and prespore regions of finger and slug structures and could be responsible for the synthesis of the extracellular cAMP that induces culmination (Verkerke-van Wijk et al., 2001). *AcaA* is expressed from three alternative promoters, specifically active at aggregation, prespore region and tip-organizer/prestalk region, respectively (Galardi-Castilla M, 2010). The activity of these promoters, and the complete promoter region, has been studied in *srfB*-mutant structures in comparison to wild-type structures. Reporter-vector driven *lacZ* expression and quantitative RT-PCR assays were used in these studies. The results obtained indicate significant differences in *acaA* expression at the tip-organizer region of the structures. Cells expressing *acaA* were more disperse in the tip region of *srfB*$^-$ finger and slug structures than in those of the wild-type strain. Similar results were obtained using reporter vectors where *lacZ* expression was under the control of the tip-specific *acaA* Promoter 3 (Fig 3) and the complete promoter region (Fig 4). In addition, an *acaA* promoter region, previously characterized as being specifically active in the tip-organizer region (23), is not functional in *srfB*-mutant structures. These results could indicate that *acaA* is not properly expressed at the tip-organizer region of *srfB*$^-$ structures, which could contribute to defective initiation of culmination.

A second difference observed was that *acaA* expression in the prespore region is much lower in *srfB*$^-$ than in wild-type structures, as indicated by reporter vectors analysis. Quantitative RT-PCR are also in agreement with this observation since the *srfB*$^-$ strain expressed significantly lower levels of both the prespore-specific mRNA (mRNA2) and total *acaA* mRNA from 8-10 hours of development, when finger and slug structures are formed. Lower expression

of *acaA* in the prespore region could contribute to a decrease in cAMP synthesis in this region. cAMP produced in the prespore region could diffuse to the tip-organizer region, contributing to induce culmination, and this signal could also be decreased in *srfB*-mutant structures.

The difference in *acaA* expression at the prespore region was not expected because *srfB* is not expressed at detectable levels in this region at the finger and slug stages of development (Galardi-Castilla M, 2008). The activity of SrfB on *acaA* expression in this region could be dependent on differences in intercellular signalling mechanisms originated in *srfB*-expressing cells, mainly located at the tip region of the structures. This possibility was studied by analyzing *acaA* expression in chimeric AX4/*srfB*$^-$ structures. The results obtained indicate that *srfB*- cells are able to repress *acaA* expression in prespore AX4 cells (Fig 5). The simplest explantion for these results would be the secretion by tip-region cells of some signalling molecules that repress *acaA* expression in prespore cells. The expression of these signalling molecules would be repressed in the presence of SrfB. According to this hypothesis, repressor molecules could be expressed at early developmental stages but would be silenced upon *srfB* expression at the tip-organizer region. *SrfB* expression would then be permissive for *acaA* expression in the prespore region, which could be required for progression through development, including inititation of culmination. These signalling mechanims are very important for coordination of development and of prespore and prestalk cells differentiation. For example, the role of signalling molecules such as DIF-1 that is produced by prespore cells and induce gene expression and differentiation in prestalk cells is well characterized (Berks and Kay, 1990). However, the results described in this article would be one of the first evidences of the existence of signalling mechanisms originated in prestalk cells and that regulate gene expression in prespore cells.

Differences in *acaA* expression were also observed during the first hours of development. In particular, *acaA* was expressed earlier in the *srfB*$^-$ strains, as detected by quantitative RT-PCR analyses of mRNA1 and total *aca*A mRNA (Fig 6). These data are in agreement with the shorter time required for silencing of growth-specific genes and induction of aggregation genes previously described for the *srfB*$^-$ strain (Galardi-Castilla M, 2008). Early induction of *acaA* expression could produce differences in cAMP expression and signalling that could contribute to the impaired aggregation observed in *srfB*$^-$ strains. However, this strain also showed defects in cell motility, adhesion and chemotaxis towards cAMP that would be independent of *acaA* expression.

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REFERENCES

Adachi, H., Hasebe, T., Yoshinaga, K., Ohta, T., and Sutoh, K. (1994). Isolation of Dictyostelium discoideum cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* **205,** 1808-1814.

Berks, M., and Kay, R. R. (1990). Combinatorial control of cell differentiation by cAMP and DIF-1 during development of Dictyostelium discoideum. *Development* **110,** 977-984.

Chisholm, R. L., and Firtel, R. A. (2004). Insights into morphogenesis from a simple developmental system. *Nat. Rev. Mol. Cell Biol.* **5,** 531-541.

Dormann, D., Vasiev, B., and Weijer, C. J. (2000). The control of chemotactic cell movement during Dictyostelium morphogenesis. *Phil. Trans. R. Soc. Lond. B* **355,** 983-991.

Escalante, R., Moreno, N., and Sastre, L. (2003). *Dictyostelium discoideum* developmentally regulated genes whose expression is dependent on the MADS-box transcription factor SrfA. *Eukaryotic Cell* **2,** 1327-1335.

Escalante, R., and Sastre, L. (2006). Investigating gene expression: In situ hybridization and reporter genes. *In* "Dictyostelium discoideum protocols" (L. Eichinger and F. Rivero, Eds.), Vol. 346, pp. 230-247. Humana Press, Totowa, NJ.

Escalante, R., Yamada, Y., Cotter, D., Sastre, L., and Sameshima, M. (2004). The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during *Dictyostelium* sporulation. *Mechanisms of Development* **121,** 51-56.

Esch, R. K., and Firtel, R. A. (1991). cAMP and cell sorting control the spatial expression of a developmentally essential cell-type-specific ras gene in Dictyostelium. *Genes Devel.* **5,** 9-21.

Fukuzawa, M., Hopper, N., and Williams, J. (1997). cudA: A Dictyostelium gene with pleiotropic effects on cellular differentiation and slug behaviour. *Development* **124,** 2719-2728.

Fukuzawa, M., and Williams, J. G. (2000). Analysis of the promoter of the cudA gene reveals novel mechanisms of Dictyostelium cell type differentiation. *Development* **127,** 2705-2713.

Galardi-Castilla M, G. A., Suarez T, Sastre L. (2010). The Dictyostelium discoideum acaA gene is transcribed from alternative promoters during aggregation and multicellular development. *PLoS One* **5,** e13286.

Galardi-Castilla M, P. B., Bloomfield G, Skelton J, Ivens A, Kay RR, Bozzaro S, Sastre L. (2008). SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in Dictyostelium. *Dev Biol. 2008* **316,** 260-74.

Harwood, A. J., Hopper, N. A., Simon, M. N., Driscoll, D. M., Veron, M., and Williams, J. G. (1992). Culmination in Dictyostelium is regulated by the cAMP-dependent protein kinase. *Cell* **69,** 615-624.

Jin, T., and Hereld, D. (2006). Moving towards understanding eukaryotic chemotaxis. *Eur. J. Cell Biol.* **85,** 905-913.

Konijn, T. M., van de Meene, J. G. C., Bonner, J. T., and Barkley, D. S. (1967). The acrasin activity of adenosine-3',5'-cyclic phosphate. *Proc. Natl. Acad. Sci. USA* **58,** 1152-1154.

Kriebel PW, P. C. (2004). Adenylyl cyclase expression and regulation during the differentiation of Dictyostelium discoideum. **56,** 541-6.

Mann, S. K. O., and Firtel, R. A. (1993). cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during Dictyostelium development. *Development* **119,** 135-146.

Pang, K. M., Lynes, M. A., and Knecht, D. A. (1999). Variables controlling the expression level of exogenous genes in Dictyostelium. *Plasmid* **41,** 187-197.

Raper, K. B. (1940). Pseudoplasmodium formation and organization in Dictyostelium discoideum. *J. Elisha Mitchell Sci. Soc.* **56,** 241-282.

Saran S, M. M., Alvarez-Curto E, Weening KE, Rozen DE, Schaap P. (2002). cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection. *J Muscle Res Cell Motil* **23,** 793-802.

Schulkes, C., and Schaap, P. (1995). cAMP-dependent protein kinase activity is essential for preaggregative gene expression in Dictyostelium. *FEBS Lett.* **368,** 381-384.

Shaulsky, G., and Loomis, W. F. (1993). Cell type regulation in response to expression of ricin-A in Dictyostelium. *Dev. Biol.* **160,** 85-98.

Smith, E., and Williams, K. (1980). Evidence for tip control of the "slug/fruit" switch in slugs of Dictyostelium discoideum. *J. Embryol. Exp. Morphol.* **57,** 233-240.

Verkerke-van Wijk, I., Fukuzawa, M., Devreotes, P. N., and Schaap, P. (2001). Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression. *Dev. Biol.* **234,** 151-160.

Wang HY, W. J. (2010). Identification of a target for CudA, the transcription factor which directs formation of the Dictyostelium tip organiser. *Int J Dev Biol* **54,** 161-5.

Williams, J. G. (2006). Transcriptional regulation of Dictyostelium pattern formation. *EMBO Rep.* **7,** 694-698.

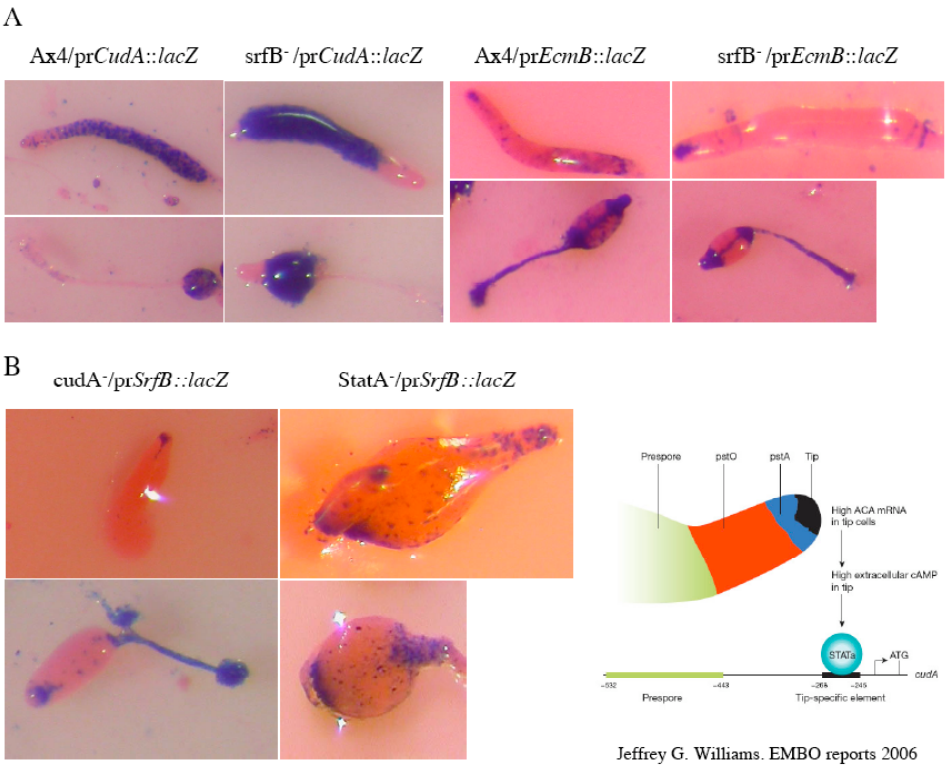

Fig 1

Figure 1. Analysis of culmination-related genes in mutant strains.

Ax4, *srfB*$^-$, *cudA*$^-$ and *statA*$^-$ strains were transfected with reporter vectors where the expression of a *lacZ* gene coding short-lived β-galactosidase was under the control of the complete promoter of *srfB* (pr*srfB*), *cudA* promoter (pr*cudA*) or *ecmB* promoter (pr*ecmB*). Transformed cells were allowed to enter multicellular development and *lacZ* expression determined by X-gal hydrolysis. A) Activity of *cudA* and *ecmB* promoters in wild type (Ax4) and *srfB* mutant cells (*srfB*$^-$) structures. B) Activity of *srfB* promoter in *cudA*$^-$ and *statA*$^-$ strains. Structures were stained with eosine after incubation with X-gal and observed under a Leica stereomicroscope.

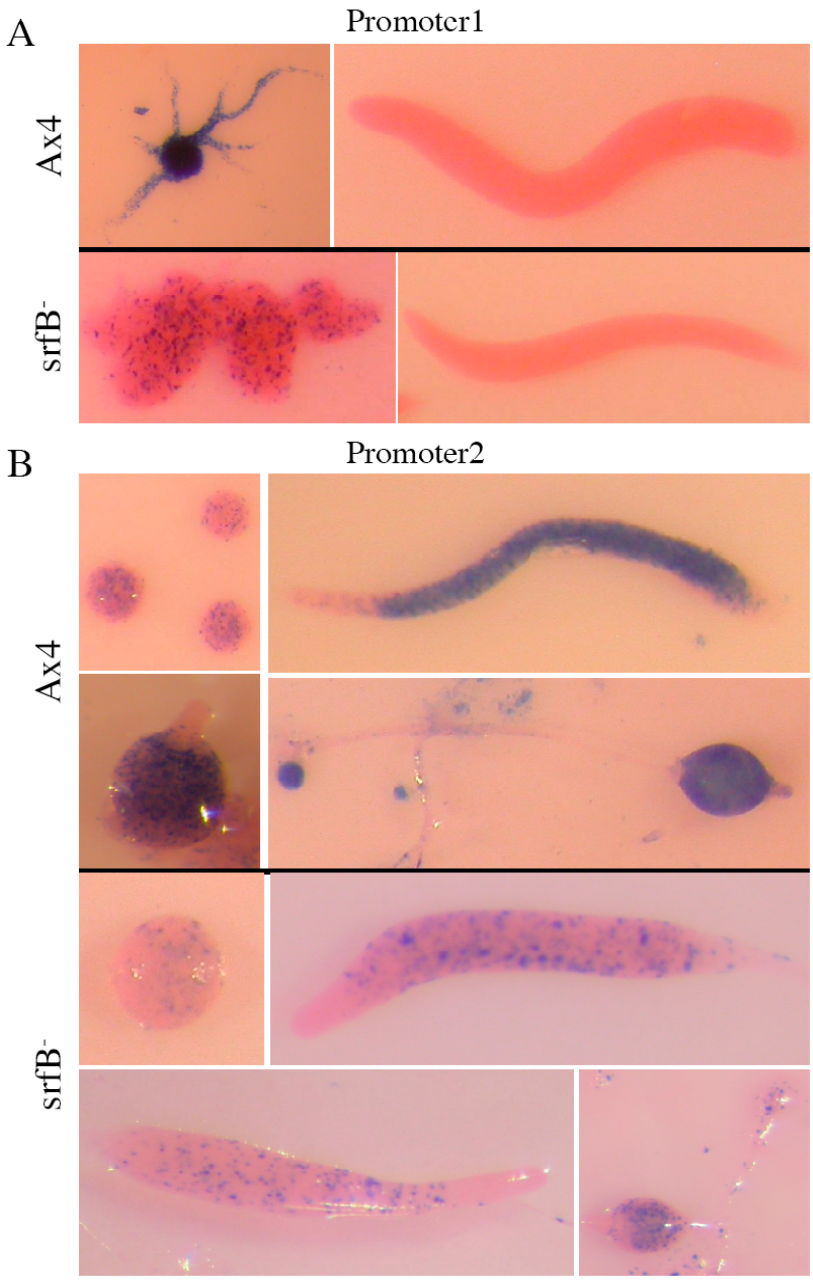

Figure 2

Figure 2. Activity of *acaA* Promoters 1 and 2 during development of Wild-type and *srfB*$^-$ structures.

Ax4 and *srfB*$^-$ strains were transfected with reporter vector where the expression of a *lacZ* gene coding short-lived β-galactosidase was under the control of *acaA* promoters 1 or 2. Transformed cells were allowed to enter multicellular development and *lacZ* expression determined by X-gal hydrolysis. A) Activity of *acaA* promoter 1 in wild type (Ax4) and *srfB* mutant cells (*srfB*$^-$) at aggregation (left panels) and slug (right panels) stages of development. B) Activity of *acaA* promoter 2 in wild type (Ax4) and *srfB* mutant cells (*srfB*$^-$) at mound (upper left panels), slug (upper right panels), early culminant (lower left panels) and culminant (lower right panels) stages of development.

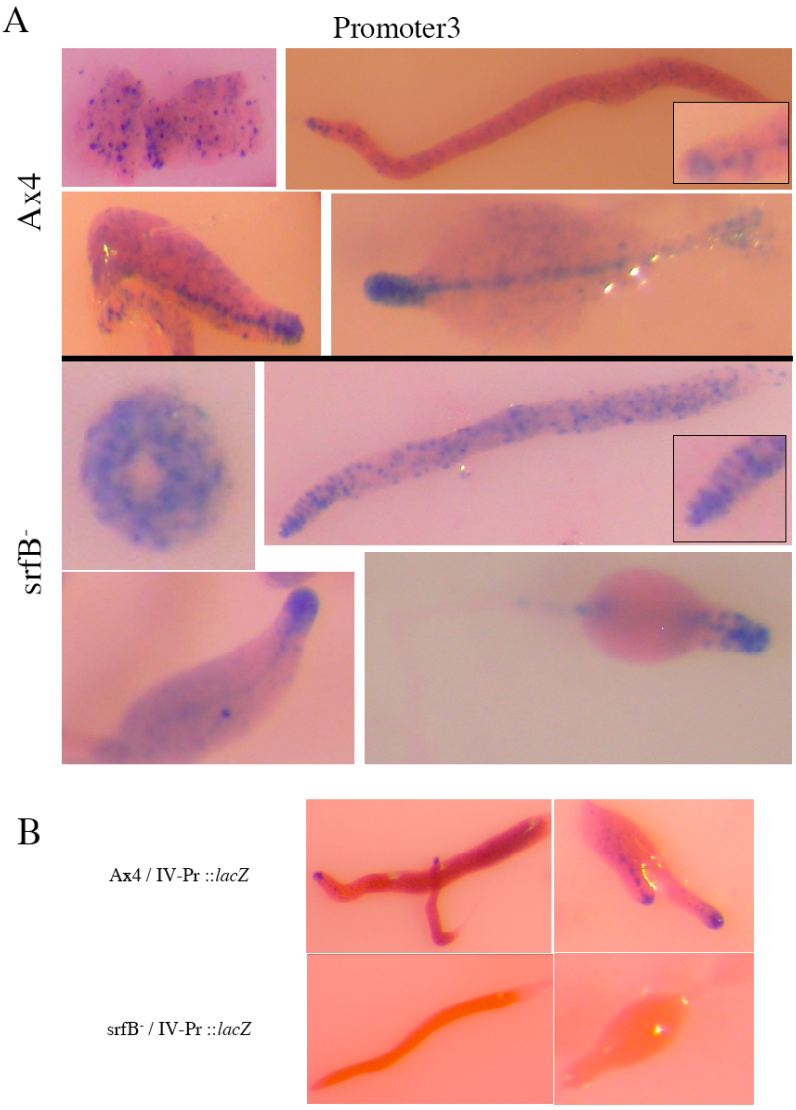

Figure 3

Figure 3. Activity of *acaA* Promoter 3 during development of Wild-type and *srfB*$^-$ structures

A. Ax4 and *srfB*$^-$ strains were transfected with a reporter vector where the expression of a *lacZ* gene coding short-lived β-galactosidase was under the control of *acaA* promoter 3. Transformed cells were allowed to enter multicellular development and *lacZ* expression determined by X-gal hydrolysis. Activity of *acaA* promoter 3 was shown in wild type (upper group of panels) and *srfB* mutant cells (*srfB*$^-$, lower group of panels), at aggregation or mound (upper left panels), slug (upper right panels), early culminant (lower left panels) and culminant (lower right panels) stages of development. Magnified pictures of the anterior part of the structures are shown in slug stage panels. **B.** Ax4 and *srfB*$^-$ strains were transfected with reporter vector where the expression of a *lacZ* gene was under the control of a previously described *acaA* gene promoter construct (IV-Pr::lacZ)(23). Transformed cells were allowed to enter multicellular development and *lacZ* expression determined by X-gal hydrolysis in wild type (upper panels) and *srfB* mutant structures (lower panels) at slug and mid culminant stages.

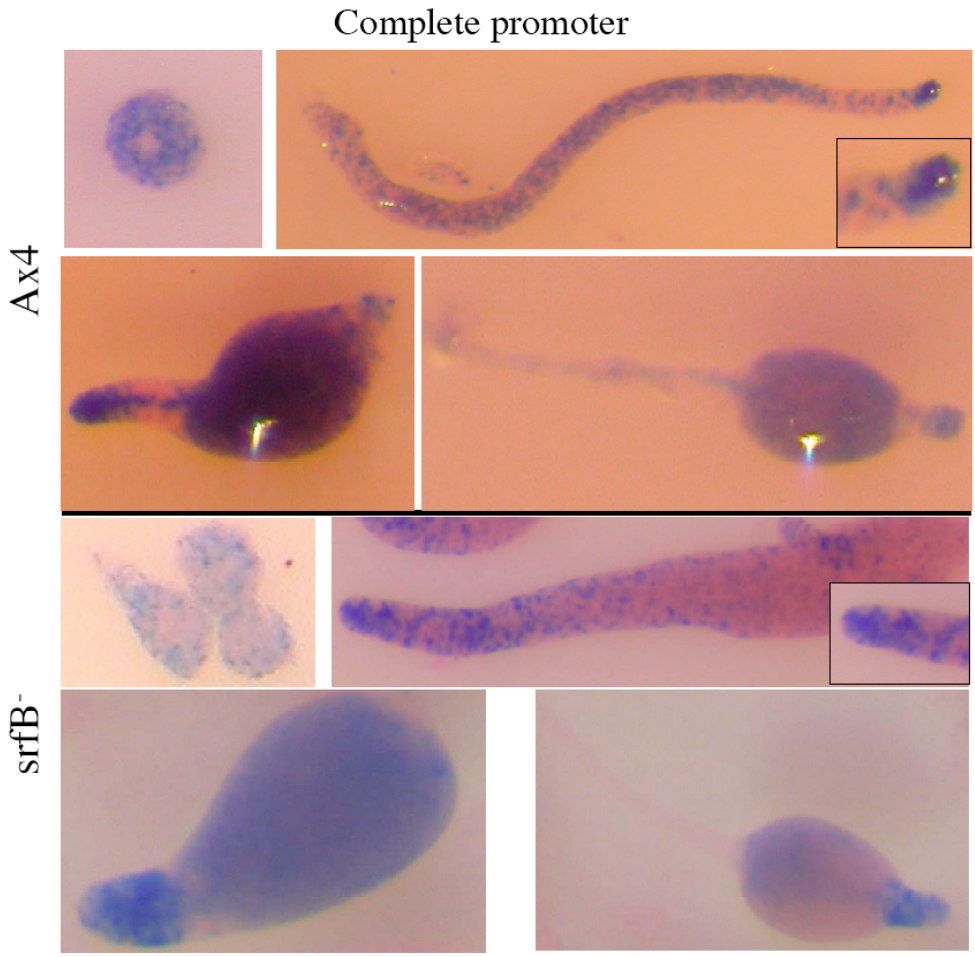

Figure 4

Figure 4. Activity of the complete *acaA* promoter during development of Wild-type and *srfB*$^-$ structures.

Ax4 and *srfB*$^-$ strains were transfected with a reporter vector where the expression of a *lacZ* gene coding short-lived β-galactosidase was under the control of the complete *acaA* promoter, covering most of the *lsm2/acaA* intergenic region and including Promoters 1, 2 and 3. Transformed cells were allowed to enter multicellular development and *lacZ* expression determined by X-gal hydrolysis. Activity of complete *acaA* promoter is shown in wild type (upper group of panels) and *srfB* mutant cells (*srfB*$^-$, lower group of panels), at mound (upper left panels), slug (upper right panels), early-culminant (lower left panels), and culminant (lower right panels) stages of development. Enlargements of the anterior part of the structures are shown in the slug stage panels.

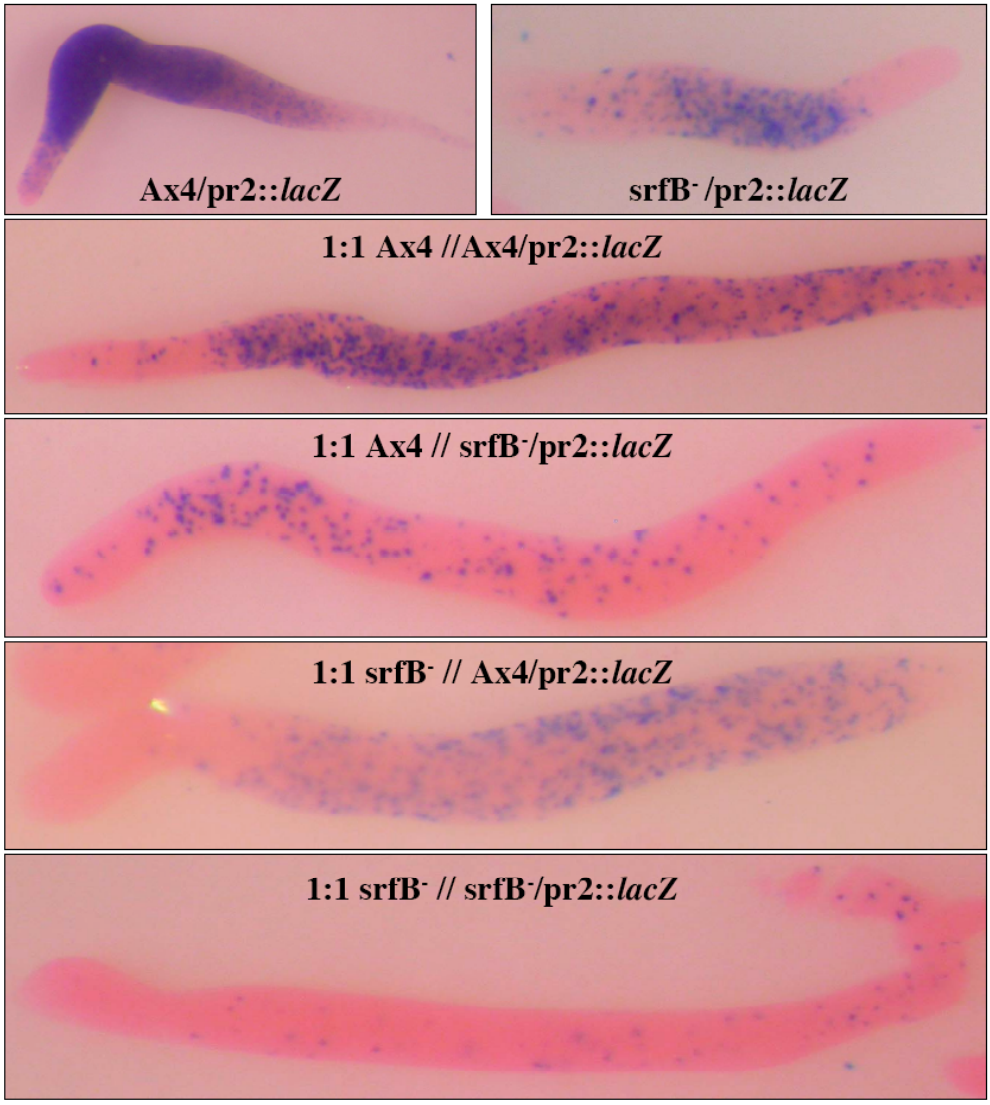

Figure 5

Figure 5. Analyses of the activity of *acaA* promoter 2 in AX4/*srfB*$^-$ chimeras.

Wild type (Ax4) or *srfB*-mutant cells (*srfB*$^-$) were mixed in a 4:1 proportion with wild-type of mutant cells transfected with reporter vectors expressing a *lacZ* gene coding short-lived β-galactosidase under the control of the *acaA* promoter 2 (pr2::*lacZ*). Cell mixtures were allowed to develop on Nitrocellulose filtres to the slug stage, collected and *lacZ* expression determinated by X-gal hydrolysis.

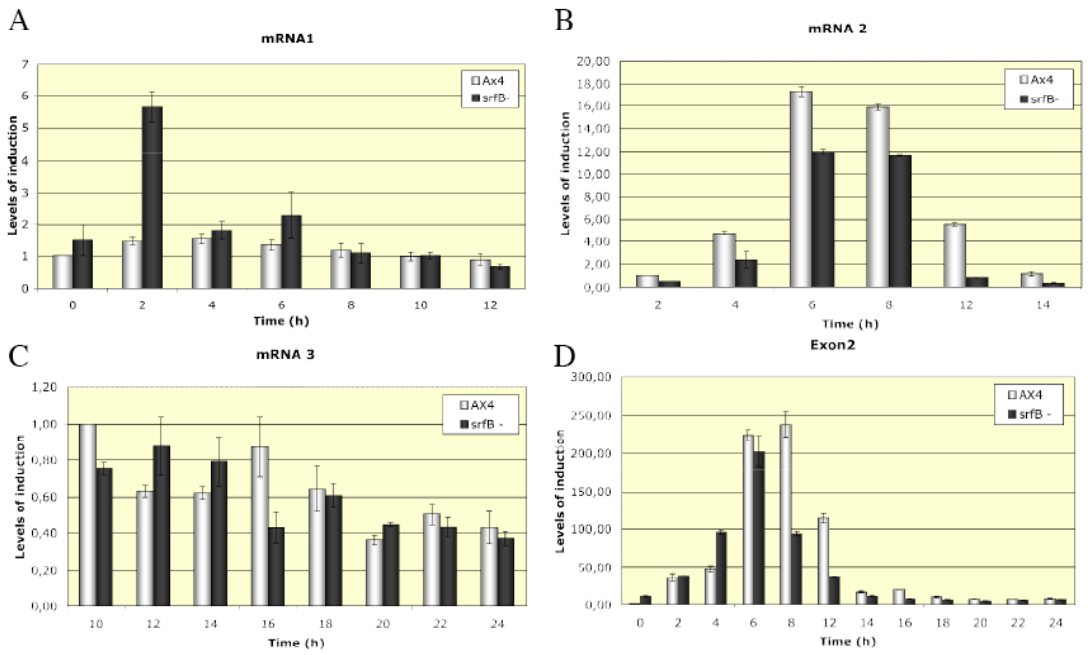

Figure 6

Figure 6. Quantification of the expression of the different *acaA* mRNAs in wild-type and *srfB* mutant cells.

RNA was extracted from wild type (AX4) or *srfB*-mutant cells (*srfB*$^-$) at growth (time 0) or from structures developed for indicate times on Nitrocellulosa filtres (time 2-24). Expression of the different *acaA* mRNAs (mRNA1, 2 y 3) was analyzed by quantitative RT-PCR using oligonucleotides specific for the 5' region of the three mRNAs. Oligonucleotides that amplify an exon 2 region common to the three mRNAs were used to estimate total *acaA* mRNA expression (Exon2). The relative value of 1 was assigned to the expression level of AX4 growing cells (time 0) in each panel. Open bars correspond to wild-type RNAs and black bars to *srfB*-mutant RNAs.

Capítulo 4: *Mef2A, homologous to animal Mef2 transcription factors, regulates cell differentiation in Dictyostelium discoideum*.

El factor inductor de miocitos 2 o "*Myocyte enhancer factor 2"* de mamíferos (Mef2) se encarga de la diferenciación celular en muchos y variados tejidos. En *Dictyostelium discoideum* se encontraron dos posibles homólogos a *Mef2*, srfC y srfD. En este artículo hemos estudiado la estructura del gen *srfC*, denominado *mef2A* en adelante. Hemos determinado que este gen se expresa a niveles bajos en células en crecimiento y se induce a partir de las 4 horas de desarrollo desde dos promotores diferentes aunque los dos ARNm generados codifican la misma proteína. El promotor más distal con respecto a la región codificante del gen se expresa específicamente en las células pre-espora, mientras que el más proximal se expresa en un pequeño grupo de células que forman parte del pie de la estructura. Hemos generado cepas mutantes para *mef2A* en la que una parte del gen ha sido sustituida por un gen de resistencia a blasticidina. Las cepas mutantes crecen más despacio que las células silvestres cuando se alimentan de bacterias. También muestran alteraciones del proceso de desarrollo; las células mutantes forman estructuras más pequeñas, aunque su morfología es similar a la de las estructuras silvestres. Además, las cepas mutantes para *mef2A* forman estructuras migratorias muy pequeñas y menos esporas viables que las cepas silvestres, aproximadamente la mitad. La expresión de marcadores específicos de los dos tipos principales de células que se forman durante el desarrollo indicó que las cepas mutantes forman más células de tallo y menos esporas que las cepas silvestres. En concordancia con estas observaciones, cuando se mezclan células mutantes para *mef2A* con células silvestres, casi todas las esporas formadas provienen de las células silvestres. Estos experimentos de mezclas también demostraron que las células mutantes no contribuyen a la formación de estructuras pre-tallo tales como el organizador del *"tip"* y la región anterior del *"tip"*. Las células mutantes no son capaces de diferenciarse a esporas *in vitro*. Estudios de expresión génica por secuenciación masiva de ARNm's aislados de estructuras de 16 horas de desarrollo indican que las estructuras mutantes expresan mayores niveles de genes específicos de células pre-tallo y menores niveles de genes de pre-espora que la cepa silvestre. Todos estos resultados refuerzan la conclusión de que mef2A participa en el proceso de determinación y diferenciación de las células pre-espora y de una población de células pre-tallo. Resulta interesante observar que el factor Mef2A desempeñe un papel en procesos de diferenciación celular, al igual que ocurre con los factores Mef2 en animales.

Mef2A, homologous to animal Mef2 transcription factors, regulates cell differentiation in *Dictyostelium dicoideum*.

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SUMMARY

Transcription factors from the MADS-box family play very relevant roles in cell differentiation and development and include the animal SRF (serum response factor) and MEF2 (myocyte enhancer factor 2) proteins. The social amoeba *D. discoideum* contains four genes coding for MADS-box transcription factors, two of them code for proteins more similar to SRF and the other two for proteins more similar to MEF2 animal factors. The biological function of one of the two genes coding for MEF2-related proteins, named *mef2A*, is described in this article. This gene is expressed in growing cells but its expression is greatly induced during development in prespore cells under the transcriptional control of two alternative promoters. The generation of mutants where the *mef2A* gene has been partially deleted, in two different strains, has allowed the study of its biological function. Mutant strains showed reduced growth when feeding on bacteria. Mutant structures developed into fruiting body but spore production was significantly reduced. Study of developmental markers showed that prespore cells differentiation was impaired in the mutants and that mutant cells formed a very low proportion of spores when cultured in conjunction with wild-type cells. In vitro spore differentiation was almost completely absent in mutant cells. In addition, mutant cells also show a poor contribution to formation of the tip-organizer and upper region of slugs and culminant developmental structures. In agreement with these observation, a comparison of the genes transcribed by mutant and wild-type strains during development indicated that the mutants expressed higher levels of several prestalk genes and lower levels of prespore genes that wild-type strains. These data indicate that *mef2A* plays a role in cell differentiation in *D. discoideum* and, in particular in prespore cell differentiation, similarly to the known function of other MADS-box transcription factors in other biological systems.

INTRODUCTION

Mef-2-related transcription factors belong to a family of proteins that is present in all eukaryotic organisms (Gramzow et al., 2010; Theiben et al., 1996). These proteins share a very conserved DNA-binding and protein-dimerization domain, the MADS-box, named after the transcription factors MCM1 from yeast, Agamous, Deficiens, from plants and SRF from animals (Shore and Sharrocks, 1995) (Treisman, 1995). The 60 amino acids long MADS-box is responsible for binding to the minor groove of the DNA through the N-terminal basic region and a contiguous α-helix region. The rest of the MADS-box is involved in stabilizing DNA binding and in the formation of dimmers, the functional structure of these proteins. Dimerization also requires a small protein region C-terminal to the MADS-box. Comparison of the amino acid sequences of the MADS-box allowed the identification of two sub-families of factors, denominated Type I and TypeII (Becker and Theissen, 2003). Plants have a large number of Type I and Type II MADS-box proteins while other organisms, like fungi or animals usually have one or a few proteins of each sub-family (Messenguy and Dubois, 2003). For example, in animals there is only a Type I protein, SRF (Serum Response Factor) and four Type II proteins, Mef2 A-D (Myocyte Enhancer Factor 2). The two types of factors recognize different A/T rich binding sites and do not dimerize between them. For example, SRF and related factors recognize the consensus sequence $CC(A/T)_6GG$ (Pollock and Treisman, 1990; Sun et al., 2007) while Mef-2 related factors recognize the $CTA(A/T)_4TAG$ consensus sequence (Black and Olson, 1998).

MADS-box transcription factors accomplish different biological functions. In plants are critically involved in floral formation and development (Becker and Theissen, 2003). In yeasts, MCM1, for example, participates in the regulation of pheromone expression, metabolism (Treisman and Ammerer, 1992) and, also, DNA replication (Chang et al., 2004). In animals, these transcription factors are mainly involved in the regulation of cell-differentiation processes. SRF deletion is lethal in mice because cells are impaired in cell adhesion and migration (Schratt et al., 2002) and the embryo cannot complete gastrulation (Arsenian et al., 1998). Tissue-specific deletion has shown that SRF is also required for terminal differentiation of skeletal, cardiac and smooth muscle cells and for neural cells migration (reviewed in (Miano, 2010)). Additional studies have demonstrated that SRF regulates the expression of a large number genes coding for actin-cytoskeleton related proteins (Miano et al., 2007; Olson and Nordheim, 2010) and, because of that, is required for cell adhesion and migration, as mentioned above.

Mef-2 proteins are also involved in regulating the expression of muscle-specific genes, both in Drosophila (Sandmann et al., 2006) and in mammals (Naya and Olson, 1999), in collaboration with MyoD-related transcription factors (Molkentin and Olson, 1996). In mammals there are four genes coding for very similar Mef-2 factors that seems to complement

each other which has made more difficult to study their biological functions through the generation of mutant mice(McKinsey et al., 2002). However, Mef2C null mice die early in development due to cardiovascular abnormalities (Lin et al., 1997) and Mef2a null mice die perinatally from heart defects (Naya et al., 2002). In addition, numerous studies have shown that Mef-2 factors are also involved in the differentiation of several cells types, in addition to muscle cells, like neural crest cells, endothelial cells, chondrocytes, neurons or lymphocytes (Arnold et al., 2007; Potthoff and Olson, 2007).

Our group has approached the functional study of MADS-box transcription factors in the social amoeba *Dyctyostelium discoideum*. These unicellular organisms life in forestal soils, feeding on bacteria and other microorganisms. In addition, they are able to develop as multicellular organisms under starving conditions. In this case, up to 10^5 individual amoeba aggregate together to form a fruiting body composed by a basal disk, a stalk and, on top of it, a sorus where up to 80% of the original amoeba differentiate into resistance forms called spores. This developmental process takes about 24 hours and can be easily reproduced in the laboratory (for recent reviews, (Schaap, 2011; Urushihara, 2011; Williams, 2010). The initial step is the aggregation of the cells towards cAMP-secreting centers to form a mound. Cells then secrete a extracellular matrix that covers the mound and initiate a differentiation process to form two main cell types: prestalk and prespore cells. Prestalk cells migrate to the top of the mound, emerging as a tip. Later on, the culmination process is initiated by the migration of prestalk cells from the tip towards the substrate through the mass of prespore cells. At the stalk, prestalk cells differentiate, elongating, secreting a cellulose extracellular coat and dying. During culmination prestalk cells continuously incorporate to the upper region of the stalk, that gets elongated. The mass of prespore cells remains attached to the top of the forming stalk, rising from the substrate until culmination gets completed. Migratory structures, called slugs, can be formed before initiation of culmination under adverse environmental conditions. In this case, slugs migrate looking for warmer and lighter places for culmination to facilitate dissemination of the spores. By the end of culmination prespore cells differentiate inside the sorus, secreting a spore coat made of proteins and cellulose, and get dehydrated to form mature spores. These resistance forms remain viable for weeks and are dispersed through the environment. When a spore reaches a place with favorable conditions gets rehydrated and germinates, breaking the spore coat and emerging as a free amoeba. *D. discoideum* fruiting body formation is being studied in some detail because it represents a simplified version of a developmental process where a simple structure is formed by two main cell types, prespore and prestalk cells, although several sub-types of prestalk cells have been identified in the last years (Williams, 2010). Despite its simplicity, mechanisms of cell migration, adhesion, intercellular signaling, morphogenesis, cell type specification and cell differentiation similar to those involved in more complex developmental processes are required.

The nucleotide sequence of *D. discoideum* genome has been determined, showing that this organisms contains four genes coding for MADS-box transcription factors, named *srfA*, B, C and D. Previous studies from our laboratory have shown that *srfA* is required for development of the fruiting body, being essential for spore terminal differentiation (Escalante and Sastre, 1998; Escalante et al., 2004) although *srfA* mutant strains also show defects in slug migration and in the culmination process (Escalante et al., 2001). *srfB* is expressed earlier than *srfA* during development and the encoded protein is involved in the initiation of the developmental process, cell migration and the initiation of culmination (Galardi-Castilla et al., 2008).

The functional study of the *srfC* gene is described in this article. The comparison of the amino acids sequences of the MADS-box regions has shown that *srfC*, and *srfD*, code for type II proteins, more similar to animal Mef-2 than to SRF transcription factors. On the other hand, *D. discoideum srfA* and *srfB* genes encode proteins more similar to SRF. *srfC* expression is induced from 4 hours of development, first in most of the cells and later on in prespore cells under the control of two alternative promoters. Mutant strains have been generated where a region of the *srfC* gene has been deleted. The analyses of these strains indicated that the encoded protein, *SrfC*, that we propose to name Mef2A, is involved in cell type specification or differentiation during *D. discoideum* development. These data further indicate that, similar to what happens in other organisms, MADS-box proteins are involved in cell differentiation and development in the social amoeba *D. discoideum*.

MATERIALS AND METHODS

Cell culture, transformation and development

D. discoideum cells were cultured axenically in HL5. Transformation by electroporation was performed as described (Pang et al., 1999). Transformed cells were selected by treatment with blasticidin (Adachi et al., 1994) or neomycin (G418). Filter development was induced by spreading 1-2 $x10^7$ cells (0.6-1.2 x 10^6 cells/cm^2) on Nitrocellulose filters (Milipore Co., Bradford, MA, USA)(Shaulsky and Loomis, 1993). Submerged development was induced by incubation of the cells in PDF phosphate-based buffer on cell-culture dishes, at $5x10^5$ cells/ml.

Phylogenetic studies

The amino acid sequences of MADS-box regions from different organisms were obtained from public databases and compared to those of the four *D. discoideum* proteins coding for MADS-box-related sequences, obtained from the DictyBase (http://www.dictybase.org). Amino acid sequences were compared using the Clustal W programs at the online Biology Workbench facilities from the San Diego Supercomputer Center (http://workbench.sdcs.edu) and the Clustal X program (Thompson et al., 1997). Phylogenetic trees were determined using the neighbour-joining method (Saitou and Nei, 1987). A random generator seed of 111 and 1000 bootstrap trials were calculated. Trees were drawn using the njplot program.

Rapid amplification of cDNA ends

RNA was isolated from AX4 cells at 8 hours of multi-cellular development on Nitrocellulose filters. The SMART ™ cDNA amplification kit from Clontech (Clontech Laboratories, Inc, Montain View, CA, USA) was used to amplification of the 5' un-translated region of *mef2A* mRNA according to the manufacture's instructions. The oligonucleotide TGTTGCCTGTCTATTTCTTTCATTAG, complementary to nucleotides 145 to 164 of the gene, was used as primer. Amplification products were cloned in the pGEMT-Easy cloning vector (Promega Co, Madison, WI, USA) and the insert of at least 10 different colonies of each product were sequenced.

Determination of *mef2A* expression by RT-PCR

RNA was isolated from $2x10^7$ cells, either at growth or after development on Nitrocellulose filters for the times indicated in each experiment, using the TRI reagent (Sigma-Aldrich, Inc, St

Louis, MO, USA) according to the manufacture's instructions. RNA was purified with RNeasy Mini kit (Quiagen). cDNAs were generated from 2 µg of total RNA using gene-specific oligonucleotides as primer. cDNAs were used as substrates for PCR reactions using as primers the oligonucleotides used for cDNA synthesis and upstream oligonucleotides from the coding region of the transcripts. Oligonucleotides GGACTAGTTTCC-ATTGAACCAATTGGGTGAGCG and CTGATAATACAGATAATACTCGC were used for *mef2A* cDNA synthesis. The large mitochondrial rRNA was amplified as a control, using oligonucleotides GGGTAGTTTGACTGGGGCGG and CACTTTAATGGG-TGAACACC.

Vectors for the generation of knockout strains

Flanking regions of the *mef2A* gene, including nucleotides -4069 to -2717 and 596 to 1298, in relation to the A of the translation initiation codon were generated by PCR and cloned at both sides of the blasticidin resistance gene in the pLPBLP plasmid vector. Oligonucleotides GGCCGCGGCCATTCCCAGCAACGCTGGTAATC, GGTCTAGACCTGGAAAACTGGAAAACCAATTG, GGATCGATCCACCCACACTAACACA-CACC and GGGTCGACGGTGGTGGTGATTGGTGCTG were used for these amplifications.

Construction of the reporter vectors

The two *mef2A* promoter regions were amplified by PCR from *D. discoideum* genomic DNA and cloned in the reporter vector PsA-ialphaGal (Detterbeck et al., 1994) in substitution of the XbaI/BglII PsA promoter fragment. Oligonucleotides GGTCTAGAGCACAAGATTATACTTGCCA and GGAGATCTCATGGTGTGTGATATG-TAAGTGAGTG were used to amplify the -2201 to -489 region of the gene, corresponding to Promoter 1. Oligonucleotides GGTCTAGACACTCACTTACATAT-CACACACC and GGAGATCTTGTTGCCTGTCTATTTCTTTCATTAG were used to amplify the -511 to 164 region, corresponding to Promoter 2. The complete promoter region (-2201 to 164) was amplified using the first and last oligonucleotides described above.

Northern Blot analyses

RNA was isolated using Trizol reagent (Gibco-BRL). RNA transfer to nylon membranes, probe labelling and hybridization were carried out as a previously described (Sambrook, 1989). DNA probes were generated by PCR using oligonucleotides designed from nucleotide sequences obtained at DictyBase (http://www.dictybase.org).

Hystochemistry and determinantion of β-galactosidase activity in developmental structures.

Cells transformed with the different reporter vectors were allowed to develop on Nitrocellulose filters for the periods of time indicated in each experiment. Structures were fixed, permeabilized and β-galactosidase activity was detected by hydrolysis of X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopiranoside) as a previously described (Escalante and Sastre, 2006).

Spore differentiation experiment

Growing cells were collected by centrifugation and re-suspended in phosphate-based PDF buffer containing 5µl/ml of Cell tracker ™ Blue CMHC (4-chloromethyl-7-hydroxycoumarin) (Invitrogen , Eugene Oregon USA) or a vehicle (DMSO) and incubated for 1 hour in shaking cultured. After that, cells were washed, resuspended in free PDF buffer and mixed in a 1:1 proportion to spreading 6,6 x 10^6 cells on Nitrocellulose filters during 24-36 hours. After that time, several sorus of each mix were harvested and dissociated in water. Spores were visualized in a Zeiss Axiophot fluorescence microscope.

Determination of mRNA levels by quantitative RT-PCR

RNA was isolates from 2 x10^7 cells, either at growth or after development on Nitrocellulose filters for the times indicated in each experiment, using the TRI reagent (Sigma-Aldrich, Inc, St Louis, MO, USA) according to the manufacture's instructions. RNA was purified with RNeasy Mini kit (Quiagen). cDNA's were generated from 2 µg of total purified RNA using random primers (Promega Co, Madison, WI, USA). cDNA's were used as substrate for quantitative real-time PCR reactions using the following gene-specific oligonucleotides: **HssA** gene (DDB_G0280999) GTGCTATTACCTCAATTTCAAG and GGCAACCACATGAA-CCACTTG; DDB_G0283503 gene CAAATCATTACAATCAATCACAAGTG and GGGCTACAGCAGCAACTG; **PrS1** gene (DDB_G0285863) CCAATAATTCTTTGAA-GGCCC and CAATAGCTTGGCCCATAGTAGC; **tgrF1** gene (DDB_G0292732) CCCACCATTTACTCCAATACTC and GTAGAGATGGTGTTGATGGAG; **tgrC5** gene (DDB_G0281407) GCTGGCTTAGCACTTTCATCAG and GAGACCAACGGCAGCGACAC; **pks32** gene (DDB_G0292732) CAACTCCAGTCACAACTATAGC and GATTATCA-TGAATGTGGAATGCTG; **mybC** gene (DDB_G0281563) GGTGGAGGTAAAACTGGTGC and CATCCATCCAACTAATATCACG; **DDB_G0290847** gene CAGTACTGAACAA-GCATTATCAAG and GTTAACATAACCTTGTTGAGAATC; **DDB_G0271438** gene

GTCATGAAATTGGAGATCGAAG and CATGAGATGATGTTGATTTGG; **psiI** (DDB_G0288919) GGTTGTACACTTGTACCACG and GAGGTGCTTCAAAGAGAGC; **DDB_G0285697** gene GGTAAGGCAGTTGTCAATGC and GCCTACCAGC-TGAGACTTCAGC. A region of the large mitochondrial ribosomal RNA was amplified as a loading control using the oligonucleotides CACTTTAATGGGTGAACACC, used as a reverse oligonucleotide, and GGGTAGTTTGACTGGGGCGG used as a forward oligonucleotide. The Step one plus real time PCR system (Life technologies Co - Applied Biosystems, Carlsbad, CA, USA) was used in these experiments. PCR products were labelled with Sybr-green using the Power Sybr®-Green PCR Master Mix (Applied Biosystems) reaction mix following the manufacturer's instructions. The final volume of the reaction was of 20 µl, using a 0,2 µM concentration of each primer. PCR conditions were as follows: 95ºC, 10 m; (95ºC, 15 s; 45ºC, 30 s; 62º 1 m) x 30-40 cycles.

In vitro spore differentiation

Exponentially growing cells were washed in KK2 buffer (16.5 mM KH_2PO_4, 3.9 mM K_2HPO_4, 2 mM $MgSO_4$, pH 6.2) and plated on culture dishes at a concentration of 10^6 Cell/ml in spore buffer (10 mM MOPS, 20 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.2), supplemented with 12.5 mM 8-Br-cyclic-AMP and 20 µM $CdCl_2$ (Kay, 1989) (Serafimidis and Kay, 2005). Cells were incubated in the dark for 30 hours and then observed under a TS100 Eclipse Nikon microscope (Nikon, Tokyo, Japan). Pistures were taken with a Leica DFC420 camera (Leica Microsystems, Wetzlar, Germany)

mRNA sequencing.

RNA was isolated from structures developed on Nitrocellulose for 16 hours using the TriReagent and purified with RNeasy Mini kit, as described previously. Poly(A)-containing RNA was isolated and converted to cDNA. The cDNA was fragmented, amplified by PCR and the nucleotide sequences determined using an Illumina Genome Analyzer IIx massive sequencer, at the Parque Científico de Madrid. Sequencing data were analyzed at Sistemas Genómicos, S.L. (Valencia, Spain). The generated sequences were mapped to the *D. discoideum* genome using the Tophat v1.1.3 software (Trapnell et al., 2009). Transcripts were identified and quantified using the Cufflins v1.0.3 program. Total number of reads per gene was determined using the HTSeq package (http://www-huber.embl.de). Statistic analyses of the results was made using the DESeq package (Anders and Huber), using a FDR of 0.01. A minimal difference of three times in expression levels was considered.

RESULTS

1. Characterization of the *mef2A* (*srfC*) gene.

Analyses of the *D. discoideum* genome allowed the identification of four genes coding for proteins with regions similar to the MADS-box domain, that were named *srfA*, B, C and D. The putative MADS-box region of the proteins encoded by these genes has been analyzed in more detail and compared to that of vertebrate (H. sapiens, G. gallus, X. laevis), invertebrate (D. melanogaster, A. franciscana), amoeba (E. histolytica) and fungi (S. cerevisiae) type I (SRF, MCM1, ARG1) and type II (Mef2) genes. Amino acid sequences were aligned using the Clustal W and X programs, and the multiple alignment used to calculate the phylogenetic tree shown in Figure 1. The results obtained indicate that the *D. discoideum* genes *srfA* and *srfB* code for proteins more similar to type I genes, like the animal SRF gene, than to type II genes, like Mef2. *D. discoideum srfA* and *srfB* seem to form a monophyletic group closer to animal proteins than to those of fungi. On the contrary, the proteins encoded by *D. discoideum srfC* and *srfD* are more similar to animal MEF2 than to SRF proteins. In this case, the protein encoded by *srfC* is more related to that of the amoeba E. histolytica and is also more similar to animal Mef2 proteins than to SrfD. Actually, SrfD seems to have diverged significantly from the other type II proteins analyzed. The results of this analysis centered our attention in *srfC*, as the *D. discoideum* gene more related to animal mef2 genes. Also because of these results we propose to rename the *srfC* gene as *mef2A*, name that will be used in the rest of the article.

Expression of *mef2A* at growth and during development was analyzed by RT-PCR and the results are shown in Figure 2A. Expression was detected in growing cells (time 0 in Fig2A) but a large induction was observed at 4 hours of development that was maintained at later developmental stages. The promoter region of the gene was characterized next to further determine the temporal and spatial patterns of expression. Primer-extension experiments were realized using the RACE (rapid amplification of cDNA ends) technique. The diagram of the structure of the 5' region of the gene, including the intron/exon structure, the 5' untranslated region and the transcription initiation sites is shown in Figure 2B. The data obtained indicate the existence of two introns in the 5' region of the gene and two different transcription initiation sites. There is an intron in the protein-coding region, located between the A of the translation initiation codon and the second nucleotide of the codon (T). The second intron identified is located in the 5' untranslated region of one of the two transcribed mRNAs (nucleotides -536 to -371, in relation to the A of the initiation codon). The analysis indicates the existence of two transcription initiation sites, located at nucleotides -144 and -814. Transcripts initiated and the -814 site are further processed by splicing out the -536/-371 intron.

The transcriptional activity of both promoters was analyzed by the use of reporter vectors where Promoter 1 (Pr1), Promoter 2 (Pr2) or the complete promoter region (cPr) were cloned

driving *lacZ* expression. The results obtained of determining *lacZ* expression by hystochemical methods at different developmental stages is shown in Figure 2C. Promoter 1 drove *lacZ* expression in scattered cells at the mound and first-finger stages of development but its activity markedly increased in the prespore region of slug, finger and culminant structures. Promoter 2 was more active than promoter 1 in scattered cells of aggregates and first fingers to decrease almost completely thereafter, except for a few cells in the basal disk of culminant structures. The complete promoter showed an additive pattern of expression and presented maximal activity in the prespore region of developing structures.

2. Generation of *mef2A*-deficient strains.

The study of the biological function of *mef2A* was approached through the generation of mutant strains where the gene was partially deleted by homologous recombination. The deleted region included the first two exons, coding for the 5' untranslated region of the gene, and the third exon, coding for the MADS-box domain of the protein. Several mutant clones were isolated for both AX2 and AX4 *D. discoideum* axenic strains. Figure 3A show RT-PCR analyses demonstrating the *mef2A* gene deletion in one AX4-derived clone (37) and two AX2-derived clones (2, 3). *Mef2A*-deleted strains grew slowlier than wild type ones when feeding on bacteria (Figure 3B), although no difference was observed in axenic liquid growth (data not shown).

Mutant strains completed development under starvation conditions but several differences with wild-type strains were observed, as shown in Figure 3C. The first differences were found during aggregation. Both mutant and Wild-type strains formed similar streams but those of the mutant strain fragmented more than the wild-type ones, forming more heterogeneous mounds. A second difference was that mutant strains formed less slugs, their size was greatly reduced and migrated shorter distances than the wild-type ones. Finally, mutant strains formed more culminant structures that were more heterogeneous in size than those of wild-type strains. The number of spores formed by each strain was quantified and mutant strains formed about half of the spores formed by wild-type strains (Table I).

The developmental phenotype of the mutant was further characterized by studying the expression of developmental marker genes by Northern blot (Figure 4). The expression of *acaA* (adenylyl ciclase A), induced at aggregation, and that of the prestalk genes *ecmA* and *ecmB* was similar in AX4 and the *mef2A* mutant strain. However, the expression of the prespore markers *pspA* and *cotB* was delayed in the *mef2A* mutant. The delay was of about two hours for *pspA* but was very pronounced for *cotB* (about 6 hours). In order to confirm these results AX4 and *mef2A*-mutant cells were transfected with reporter vectors that drive *lacZ* expression under the control of the *ecmA* and *ecmB* prestalk genes promoters or the *psaA* prespore gene promoter. lacZ expression was analyzed at the mound, slug and mid-culminant stages of development

(Figure 5). The *ecmA* gene promoter is active in the anterior, prestalk region of mound and slug structures and in the stalk, upper and lower cups and basal disk of wild-type structures. The activity was detected in the same regions in *mef2A* mutants except that the anterior prestalk region was larger in mounds and that *ecmA* promoter activity was more intense and extended in mutant slugs. *ecmB* promoter activity is more restricted in the anterior prestalk region of mound and slug structures than that of the *ecmA* promoter, and is mainly restricted to the tip organizer region, besides some cells scattered at the posterior regions, the anterior like cells. In culminant structures, the *ecmB* promoter is active in the complete stalk region. Mutant strains show an extended region of *ecmB* promoter activity in mound and slug structures and a well-defined tip organizer region was not observed. The *psaA* prespore promoter presented a pattern of activity complementary to that of the *ecmA* promoter. In mounds and slugs it was active in the posterior, prespore region. In culminant structures it was active in the sorus. Mef2A mutants showed very reduced *psaA* promoter activity, specially in slugs (Figure 5), but also in culminant structures where the sorus was narrower than in wild-type ones.

The amount of cells expressing each of the markers was quantified by disaggregating first-finger structures (16 hours of development) and counting the cells that expressed *lacZ*, as determined by Xgal staining. The results, shown in Table II, indicated that *ecmA* and *ecmB* promoters were active in a larger percentage of *mef2A*-mutant cells than in wild-type AX4 cells. On the contrary, the *psaA* promoter was active in a lower percentage of *mef2A*-mutant cells, as compared with the wild-type ones.

The differences observed could be due to the participation of Mef2A in the process of prespore differentiation but could also be due to defective inter-cellular signaling in the mutant structures. Experiments where mutant and wild-type cells were mixed before induction of development were designed to discriminate between these two possibilities. In the first ones, wild-type cells were mixed in a 4:1 proportion with either wild-type or *mef2A*-mutant cells that expressed *lacZ* under the control of the ubiquitous Actin 15 promoter. The distribution of *lacZ*-expressing cells in streams, slugs, finger and culminant structures is shown in Figure 6. AX4 cells expressing *lacZ* were randomly distributed at all stages of development, even if staining was more intense in stalk structures probably due to the better accessibility of the Xgal substrate. However, *mef2A*-mutant cells expressing *lacZ*, even if randomly mixed in streams, got enriched in the lower and posterior regions of slug and finger structures and in the stalk region of culminant structures, being mostly absent from prespore and spore regions of finger and culminant structures (Figure 6).

Better understanding of the differentiation capacities of *mef2A* mutant cells was intended by mixing wild-type and mutant cells expressing *lacZ* from the prestalk-specific *ecmB* promoter or the prespore-specific *psaA* promoter. A proportion of four unlabeled cells for each labeled cell was also used in these experiments. Slugs and early culminant structures were analyzed and

the results are shown in Figure 7. The described distribution of *ecmB*-expressing cells can be observed in the AX4/AX4-lacZ samples. *ecmB*-expressing cells are located in the tip-organizer region of the slugs, as well as scattered in their posterior region. In culminant structures, *ecmB* is expressed in the stalk, including the tip, and in upper- and lower-cup regions. The mixture of *mef2A* mutant labeled and unlabeled cells also show a canonical staining patter, similar to that shown in Figure 5. When *ecmB*-expressing *mef2A* mutant cells are mixed with wild-type cells, no expression was detected in the tip organizer region in slugs or in the tip of culminant structures. However, AX4-*ecmB*::lacZ cells were found in these regions when mixed with *mef2A* mutant cells.

The results obtained using *psaA*::lacZ as cell marker are shown in the lower panel of Figure 7. Homogeneous mixtures of AX4/AX4 and *mef2A*-mutant/*mef2A*-mutant cells presented the same pattern of staining shown in Figure 5. In the case of the mutant cells, a very reduced population of stained cells was also observed in slugs and, to a lesser extend, culminant structures. The mixture of *psaA*-labeled *mef2A* mutant cells with wild-type unlabeled cells showed that very few mutant cells differentiated as prespore cells and the ones that expressed *psaA* were found in the back region of the slugs and the lower part of the sorus in culminating structures. On the contrary, intense *lacZ* staining was observed when *psaA*-expressing AX4 cells were mixed with *mef2A*-mutant cells.

Mixing experiments were also used to study spore differentiation. In this case, cells were labeled by incubation with Cell-tracker and mixed in a 1:1 proportion with unlabeled cells. Cells mixtures were extended on Nitrocellulose filters and allowed to differentiate into fruiting bodies for 24 hours. Spores were collected and the percentage of fluorescent ones determined, as shown in Table III. Homogeneous mixtures of wild-type (AX4 or AX2) or *mef2A*-mutant cells yielded the expected proportion of about 50% fluorescent spores. However, the mixture of labeled wild-type cells with unlabeled *mef2A*-mutant cells produced over 90% of fluorescent wild-type spores. On the contrary, when labeled *mef2A*-mutant cells were mixed with unlabeled wild-type cells, less than 10% of the spores showed fluorescence.

Further confirmation of these results was obtained by in vitro differentiation studies. In these experiments, dissociated cells were induced to differentiate into spores by incubation with 8-Br-cAMP. After 30 hours of treatment the presence of differentiated spores was determined by microscopical observation (Figure 8). Treatment of AX4 cells induced the differentiation of most of the cells into ellipsoid, highly refringent spores. However, when *mef2A*-mutant cells were treated, most of the cells got rounded, very flattened and very few refringent spores were observed (lower panel of Figure 8). Similar results were obtained for AX2 wild-type and mutant cells (data not shown).

3. Gene expression profile of *mef2A* mutant cells.

The AX4 strain mutant for the *mef2A* transcription factor was further characterized by studying possible differences in gene expression with respect to the wild-type strain. RNAs were isolated from AX4 and *mef2A*-mutant structures collected after 16 hours of development (first finger stage). Poly(A)$^+$RNAs were isolated, converted to cDNA and sequenced using an Illumina massive sequencing machine. The sequences obtained were aligned over the AX4 genome sequence and the number of sequences obtained for each gene was determined. The genes that presented a significant difference in the number of reads between wild-type and *mef2A*-mutant strains was finally determined. Seventy seven genes, out of the about 13500 transcripts that were analyzed, showed significant differences of more than 3 times in the number of reads, with a p adjusted value of less than 0,01. Thirty two of these genes showed higher expression in the wild-type strain and forty five higher expression in the *mef2A*-mutant strain. As a control, the *mef2A* transcript was sequenced 73 times in the AX4 sample and none in the mutant sample. The more significant genes found that code for known proteins or for proteins with some conserved domains are shown in Table IV. The largest group of genes code for small proteins that are expressed in prestalk cells and that are expressed, most of them, to higher levels in the *mef2A*-mutant structures. A number of these genes show similarity to the hssA gene (Shimada et al., 2008) while a second group code for 57-59 amino acid long proteins that do not show significant similarity to HssA. There are also three genes coding for small proteins (69-72 amino acids long) that are expressed in prespore cells and that show lower expression in *mef2A*-mutant structures. Other genes that are important for prestalk development and that are expressed at higher levels in *mef2A*-mutant structures include Pks32, that code for a polyketide synthase and could be involved in the synthesis of prestalk differentiation factors, and mybC, that codes for a transcription factor involved in the response to prestalk differentiation factors (Guo et al., 1999). There are a significant number of genes that code for proteins possibly involved in transcription regulation and whose expression seems to be dependent on *mef2A* because is significantly decreased in mutant structures.

The data obtained by mRNA sequencing correspond to a single time point of the developmental process and had to be confirmed, and extended to the complete developmental process, by quantitative RT-PCR experiments. RNAs, different from those used for mRNA sequencing, were isolated from structures collected every two hours of development. The expression level of several of the genes shown in Table IV was determined by quantitative RT-PCR and the results are shown in Figure 9. The genes analyzed were representative of the main categories identified in Table IV, including genes coding for small proteins that were expressed at higher levels in the mutant (hssA, G0283503) or in wild-type strains (G0285863). Other genes analyzed included those coding for proteins involved in prestalk differentiation (Pks32, mybC). Other genes coded for proteins possibly involved in transcription regulation

(G0290847, G0271438, mybC), spore inducing factors (psiI) and membrane proteins possibly involved in extracellular signaling or cell adhesion (tgrF1, tgrC5, G0285697). The results obtained for these genes are in complete agreement with the mRNA sequencing data and show that most of these genes are similarly dependent on *mef2A* at all the developmental times analyzed. The only exception was psiI where a difference in the time of expression was observed since it was expressed at later developmental stages in *mef2A*-mutant than in wild-type structures (16 hours vs 6 hours). The expression of several of the genes analyzed (G0285863, G0290847, G0271438, G0285697) was almost completely dependent on *mef2A* at all the developmental stages analyzed.

DISCUSSION

The study of the biological function of the gene *mef2A*, coding for a protein very similar to animal Mef2 transcription factors, has been approached through the generation of a deletion mutant in *D. discoideum*. Several mutants were generated in AX2 and AX4 strains and similar phenotypes were observed for all of them. AX4 mutants have been studied in more detail but the main phenotypic characteristics have been also observed in AX2 mutants, as shown in Figure 3 and Tables I and III. Over-expression of *mef2A* caused developmental defects similar to those observed in the mutants (data not shown), which made very difficult to perform mutant complementation studies.

The results obtained indicate that *mef2A* is involved in the determination or differentiation of prespore cells and of a group of prestalk cells in *D. discoideum*. The proposed role would represent a conserved function for Mef2 proteins during evolution, since plant and animal homologous proteins also play important roles in cell differentiation processes, as mentioned in the Introduction. Several evidences support the proposed role in prespore determination or differentiation. The *mef2A* gene is expressed in the prespore region from early developmental stages, since its expression is induced at four hours of development. The expression of prespore-specific markers is decreased in *mef2A*-mutant strains, as shown by Northern blot analyses and hystochemical analyses of prespore reporter vectors. The defects observed in prespore makers expression is cell autonomous, as shown by the study of mixed populations with wild-type cells. In addition, *mef2A* mutant structures express higher levels of prestalk markers, as shown by hystochemical analyses. A second line of evidence is that *mef2A* mutants do not differentiate to spore in vitro and, in vivo, produce about half the spores than wild-type strains.

Massive-sequencing analyses of gene expression is also in agreement with this interpretation. Many of the genes whose expression decreases in *mef2A* mutants are specifically expressed in prespore cells, as determined by the mRNA expression analysis show at the Dictybase (DictyExpress, reviewed in (Loomis and Shaulsky, 2011)) and in situ hybridization analysis (Maruo, 2004). Examples are psiI (prespore inducing factor), the group of prespore-specific small proteins or tgrC5, but also a group of proteins possibly involved in transcription regulation or the putative membrane proteins shown in Table IV. A number of prespore specific proteins, often used as prespore markers, also showed differences in expression between AX4 and the *mef2A* mutant strain but did not fulfill the filter values used in the analysis of the sequencing data (more than 3 times of difference in expression level and a p value smaller than 0,01). For example, cotA was expressed 2,12 more times in AX4 than in the mutant, cotC 2,02 times, cotD 1,48, pspD 1,95 and pspB 2,52 times more.

On the contrary, many of the genes that are over-expressed in the *mef2A* mutant have been identified as perstalk- specific using the technique mentioned above. Among them are the hssA-related genes as well as the other genes that code for small proteins shown in Table IV. Two of the hssA-related genes showed higher expression in the wild-type strain in the mRNA sequencing analyses (Table IV) however, quantitative RT-PCR analyses indicated higher expression in the mutant strain at later developmental stages than the time used for mRNA sequencing, 16 hours (data not shown). Other prestalk-specific genes include the polyketide syntahse family Pks32 and the mybC transcription factor. A typical prestalk marker, ecmA, is also expressed 1,5 times more in the mutant according to the massive mRNA sequencing data while ecmB was expressed 1,5 times more in the wild-type strain at this time of development.

Mef2A seems to be also involved in the determination or differentiation of a population of prestalk cells that is located at the top of the mound and finger structures and at the anterior-most region of the slugs. These cells are characterized by the expression of the *ecmB* gene and include the tip-organizer cells that regulate culmination of the structures (Williams, 2006). The evidence for this function is that *mef2A*-mutant cells expressing *ecmB*::lacZ do not participate in the formation of the tip in mixed developmental processes (Figures 6, 7). Also, *mef2A* mutant cells differentiate poorly to *ecmB*-expressing prestalk cells in vitro (data not shown). The slightly higher expression of *ecmB* in wild-type cells shown in mRNA sequencing experiments, as mentioned above, would be also in agreement with this proposed function. We have not detected *mef2A* expression in this prestalk cells using reporter vectors. The cell-autonomous function of *mef2A* in the differentiation of these cells could be due to a cell-type determination process at the previous mound stage of development where *mef2A* seems to be expressed in a large proportion of the cells under the control of the two alternative promoters. Alternatively, expression of *mef2A* in *ecmB*-expressing prestalk cells could be too low to be detected by gene reporter expression analyses.

Differences in cell type specification could explain the phenotypes observed in the *mef2A* mutant strain. For example, the existence of a large number of prestalk cells that adhere more strongly among them could contribute to breakage of the streams. In addition, some of the genes that are miss-regulated in *mef2A* mutants, like the tgr family of genes (tgrF1, tgrC5), are involved in cell adhesion (Benabentos et al., 2009). However, no differences in Ca-dependent cell adhesion could be determined experimentally (data not shown). Slug structures also show a very altered proportion and distribution of prestalk and prespore cells (Figure 5) that could explain their smaller size and limited motility. The structure of the tip-organizer region also is very altered in the mutant slugs, as determined by the pattern of *ecmB* gene expression. This region is known to play a regulatory role in slug migration . In addition, culture under non-buffered conditions used to favor slug formation induces in the mutant strain the formation of a

large number of small mounds with many *ecmB*-expressing cells located in the basal region, which seems to impair slug formation.

Despite the developmental defects discussed above, it seems clear that *mef2A* is not absolutely required for *D. discoideum* development since a large number of fruiting bodies and spores are formed in the mutant strains. We would like to suggest that *mef2A* participates in a network of transcription factors that regulate cell differentiation and that there are other factors of the network that compensate, at least partially, the absence of *mef2A*. One of these factors could be SrfD, that is also homologous to Mef2 transcription factors. Besides SrfD, there is some evidence for the involvement of other transcriptional regulatory proteins in the process of prespore differentiation. Mutation of histone deacetylases (Sawarkar et al., 2009) and chromatin-binding proteins (Dubin et al., 2011) affects prespore differentiation or cell-type patterning. The expression of several genes coding for putative transcriptional regulators is also dependent of *mef2A*. For example, comH codes for a GATA-binding transcription factor expressed in prespore cells. G0288967 codes for a putative β-sandwich domain transcription factor and G0290847, G0290855 and G0271438 code for proteins containing domains possibly involved in DNA binding and transcription regulation. A retinoblastoma homolog, rblA also controls the preference of the cells for stalk or spore differentiation (MacWilliams et al., 2006). This gene is expressed in the prespore region and, in chimera with wild-type cells, rblA mutant cells show a strong preference for stalk differentiation, as also shown here for mef2A mutants. RblA expression is induced, however at later developmental stages than mef2A and seems to play a more relevant role in spore differentiation.

Mef2A mutants also show impaired growth when feeding on bacteria. This defect does not seem to be due to a reduced phagocytic capacity (data not shown), but has not been further characterized. However, *mef2A* (*srfC*) has been identified previously as one of the genes whose expression is regulated depending on the growth substrate of the *D. discoideum* cells, bacteria or axenic media (Sillo et al., 2008).

The activity of vertebrate Mef2 transcription factors is tightly regulated by extracellular signals. One of the best known regulatory pathways involve the association of Mef2 with class II histone deacetylases in an inhibitory complex that can be dissociated by extracellular signals through protein phosphorylation (Miska et al., 1999). Mef2 can also be directly phosphorylated through MAPkinase pathways, regulating its transcriptional activity (Han et al., 1997). *D. discoideum* codes for only two MAPkinase proteins, ErkA and ErkB and it has been described that ErkB is required for spore differentiation (Nguyen and Hadwiger, 2009; Nguyen et al., 2010). It would be interesting to determine if *mef2A* participates in this ErkB-mediated spore differentiation pathway. We have tried to complement ErkB mutant strains with constitutively active *mef2A*/VP16 fusion proteins. Unfortunately, expression of this active form in wild-type cells induces developmental defects, which invalidated the use of this experimental approach.

The initial analysis of the structure of the *mef2A* gene detected some interesting charactersistics. One of them is the presence of one intron placed between the A and the T of the translation initiation codon that could not be predicted by the informatic prediction of the structure of the gene. A second peculiarity was the existence of alternative promoters that drive the expression of the gene at different times of development and in distinct structures. It is remarkable the related *srfA* and *srfB* genes are also transcribed from alternative promoters, specific for different cell types and developmental stages (Escalante et al., 2001; Galardi-Castilla et al., 2008). Other developmental regulatory genes are also transcribed from alternative promoters in *D. discoideum*, such as pdsA (extracellular phosphodiesterase) (Faure et al., 1990) carA (cAMP receptor) (Louis et al., 1993) or acaA (adenylyl cyclase A) (Galardi-Castilla et al., 2010). The functional study of these genes indicates that the existence of alternative promoters might have been a evolutionary adaptation to regulate the participation of a given protein in different conditions of growth and/or different developmental processes, that could be also the case for *mef2A*.

Several of the genes that are under-expressed in *mef2A* mutants seem to be almost completely dependent on this transcription factor for their expression, as mentioned above. The regulation of the expression of these genes could be mediated by direct binding of *mef2A* to their regulatory regions. Alternatively, *mef2A* could regulate the expression of other transcription factors that control the expression of these genes. The DNA binding site of mef2-related factors has been conserved through evolution and corresponds to the consensus sequence CTA(A/T)$_4$ATG. We have looked for the presence of this sequence on the 1000 nucleotides long fragments located upstream of the putative *mef2A*-dependent genes but only tgrC5 presented three putative Mef2-binding sites in this region. Therefore, further experimental work will be required to determine the DNA-binding region recognized by *mef2A* and the mechanisms involved in the regulation of the expression of the possible target genes.

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REFERENCES

Adachi, H., Hasebe, T., Yoshinaga, K., Ohta, T., Sutoh, K., 1994. Isolation of Dictyostelium discoideum cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. Biochem. Biophys. Res. Commun. 205, 1808-1814.

Anders, S., Huber, W., Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Arnold, M. A., Kim, Y., Czubryt, M. P., Phan, D., McAnally, J., Qi, X., Shelton, J. M., Richardson, J. A., Bassel-Duby, R., Olson, E. N., 2007. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell. 12, 377-89.

Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., Nordheim, A., 1998. Serum response factor is essential for mesoderm formation during mouse embryogenesis. Embo J. 17, 6289-99.

Becker, A., Theissen, G., 2003. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol. 29, 464-89.

Benabentos, R., Hirose, S., Sucgang, R., Curk, T., Katoh, M., Ostrowski, E. A., Strassmann, J. E., Queller, D. C., Zupan, B., Shaulsky, G., Kuspa, A., 2009. Polymorphic members of the lag gene family mediate kin discrimination in Dictyostelium. Curr Biol. 19, 567-72.

Black, B. L., Olson, E. N., 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol. 14, 167-96.

Chang, V. K., Donato, J. J., Chan, C. S., Tye, B. K., 2004. Mcm1 promotes replication initiation by binding specific elements at replication origins. Mol Cell Biol. 24, 6514-6524.

Dubin, M. J., Kasten, S., Nellen, W., 2011. Characterization of the Dictyostelium homolog of chromatin binding protein DET1 suggests a conserved pathway regulating cell type specification and developmental plasticity. Eukaryot Cell. 10, 352-62.

Escalante, R., Sastre, L., 1998. A serum response factor homolog is required for spore differentiation in Dictyostelium. Development. 125, 3801-3808.

Escalante, R., Sastre, L., Investigating gene expression: In situ hybridization and reporter genes. In: L. Eichinger, F. Rivero, Eds.), Dictyostelium discoideum protocols, Vol. 346. Humana Press, Totowa, NJ, 2006, pp. 230-247.

Escalante, R., Vicente, J. J., Moreno, N., Sastre, L., 2001. The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of Dictyostelium development. Dev Biol. 235, 314-29.

Escalante, R., Yamada, Y., Cotter, D., Sastre, L., Sameshima, M., 2004. The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during *Dictyostelium* sporulation. Mechanisms of Development. 121, 51-56.

Faure, M., Franke, J., Hall, A. L., Podgorski, G. J., Kessin, R. H., 1990. The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains 3 promoters specific for growth, aggregation, and late development. Mol. Cell. Biol. 10, 1921-1930.

Galardi-Castilla, M., Garciandia, A., Suarez, T., Sastre, L., 2010. The Dictyostelium discoideum acaA gene is transcribed from alternative promoters during aggregation and multicellular development. PLoS One. 5, e13286.

Galardi-Castilla, M., Pergolizzi, B., Bloomfield, G., Skelton, J., Ivens, A., Kay, R. R., Bozzaro, S., Sastre, L., 2008. SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in Dictyostelium. Dev Biol. 316, 260-274.

Gramzow, L., Ritz, M. S., Theissen, G., 2010. On the origin of MADS-domain transcription factors. Trends Genet. 26, 149-53.

Guo, K., Anjard, C., Harwood, A., Kim, H. J., Newell, P. C., Gross, J. D., 1999. A myb-related protein required for culmination in Dictyostelium. Development. 126, 2813-2822.

Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., Ulevitch, R. J., 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. Nature. 386, 296-9.

Kay, R. R., 1989. Evidence that elevated intracellular cyclic AMP triggers spore maturation in Dictyostelium. Development. 105, 753-759.

Lin, Q., Schwarz, J., Bucana, C., Olson, E. N., 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science. 276, 1404-7.

Loomis, W., Shaulsky, G., 2011. Developmental changes in transcriptional profiles. Devel. Growth Differ.

Louis, J. M., Saxe III, C. L., Kimmel, A. R., 1993. Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during Dictyostelium development. Proc. Natl. Acad. Sci. USA. 90, 5969-5973.

MacWilliams, H., Doquang, K., Pedrola, R., Dollman, G., Grassi, D., Peis, T., Tsang, A., Ceccarelli, A., 2006. A retinoblastoma ortholog controls stalk/spore preference in Dictyostelium. Development. 133, 1287-97.

Maruo, T., Sakamoto, H., Iranfar, N., Fuller, D., Morio, T., Urushihara, H., Tanaka, Y., Maeda, M., Loomis, W.F., 2004. Control of cell type proportioning in Dictyostelium discoideum by differentiation-inducing factor as determined by in sity hybridization. Euk. Cell. 3, 1241-1248.

McKinsey, T. A., Zhang, C. L., Olson, E. N., 2002. MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci. 27, 40-7.

Messenguy, F., Dubois, E., 2003. Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene. 316, 1-21.

Miano, J. M., 2010. Role of serum response factor in the pathogenesis of disease. Lab Invest. 90, 1274-84.

Miano, J. M., X, L., Fujiwara, K., 2007. Serum response factor: master regulator of the actin cytoskeleton and contractil apparatus. Am J Physiol Cell Physiol. 292, C70-C81.

Miska, E. A., Karlsson, C., Langley, E., Nielsen, S. J., Pines, J., Kouzarides, T., 1999. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J. 18, 5099-107.

Molkentin, J. D., Olson, E. N., 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A. 93, 9366-73.

Naya, F. J., Black, B. L., Wu, H., Bassel-Duby, R., Richardson, J. A., Hill, J. A., Olson, E. N., 2002. Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor. Nat Med. 8, 1303-9.

Naya, F. J., Olson, E., 1999. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr Opin Cell Biol. 11, 683-8.

Nguyen, H. N., Hadwiger, J. A., 2009. The Galpha4 G protein subunit interacts with the MAP kinase ERK2 using a D-motif that regulates developmental morphogenesis in Dictyostelium. Dev Biol. 335, 385-95.

Nguyen, H. N., Raisley, B., Hadwiger, J. A., 2010. MAP kinases have different functions in Dictyostelium G protein-mediated signaling. Cell Signal. 22, 836-47.

Olson, E. N., Nordheim, A., 2010. Linking actin dynamics and gene transcription to drive cellular motile functions. Nat Rev Mol Cell Biol. 11, 353-65.

Pang, K. M., Lynes, M. A., Knecht, D. A., 1999. Variables controlling the expression level of exogenous genes in Dictyostelium. Plasmid. 41, 187-197.

Pollock, R., Treisman, R., 1990. A sensitive method for the determination of protein-DNA binding specificities. Nucleic Acids Res. 18, 6197-204.

Potthoff, M., Olson, E. N., 2007. MEF2: a central regulator of diverse developmental programs. Development. 134, 4131-4140.

Saitou, N., Nei, M., 1987. The Neighbor-Joining method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Sambrook, J., Frisch, E.F., Maniatis, T., 1989. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sandmann, T., Jensen, L. J., Jakobsen, J. S., Karzynski, M. M., Eichenlaub, M. P., Bork, P., Furlong, E. E., 2006. A temporal map of transcription factor activity: mef2 directly regulates target genes at all stages of muscle development. Dev Cell. 10, 797-807.

Sawarkar, R., Visweswariah, S. S., Nellen, W., Nanjundiah, V., 2009. Histone deacetylases regulate multicellular development in the social amoeba Dictyostelium discoideum. J Mol Biol. 391, 833-48.
Schaap, P., 2011. Evolutionary crossroads in developmental biology: Dictyostelium discoideum. Development. 138, 387-96.
Schratt, G., Philippar, U., Berger, J., Schwarz, H., Heidenreich, O., Nordheim, A., 2002. Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. The Journal of Cell Biology. 156, 737-750.
Serafimidis, I., Kay, R. R., 2005. New prestalk and prespore inducing signals in Dictyostelium. Dev Biol. 282, 432-41.
Shaulsky, G., Loomis, W. F., 1993. Cell type regulation in response to expression of ricin-A in Dictyostelium. Dev. Biol. 160, 85-98.
Shimada, N., Kanno-Tanabe, N., Minemura, K., Kawata, T., 2008. GBF-dependent family genes morphologically suppress the partially active Dictyostelium STATa strain. Dev Genes Evol. 218, 55-68.
Shore, P., Sharrocks, A. D., 1995. The MADS-box family of transcription factors. Eur J Biochem. 229, 1-13.
Sillo, A., Bloomfield, G., Balest, A., Balbo, A., Pergolizzi, B., Peracino, B., Skelton, J., Ivens, A., Bozzaro, S., 2008. Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in Dictyostelium. BMC Genomics. 9, 291.
Sun, Q., Chen, G., Streb, J. W., Long, X., Yang, Y., Stoeckert Jr, C. J., Miano, J. M., 2007. Defining the mammalian CArGome. Genome Res. 16, 197-207.
Theiben, G., Kim, J. T., Saedler, H., 1996. Classification and Phylogeny of the MADS-box Multigene Family Suggest Defined Roles of MADS-box Gene Subfamilies in the Morphological Evolution of Eukaryotes. J mol evol. 43, 484-516.
Thompson, J., Gibson, T., Plewniak, F., Jeanmougins, F., Higgins, D., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876-4882.
Trapnell, C., Pachter, L., Salzberg, S. L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 25, 1105-11.
Treisman, R., 1995. DNA-binding proteins. Inside the MADS box. Nature. 376, 468-9.
Treisman, R., Ammerer, G., 1992. The SRF and MCM1 transcription factors. Curr Opin Genet Dev. 2, 221-6.
Urushihara, H., 2011. Social amoeba and the origin of multicellularity. Dev Growth Differ. 53, 451.
Williams, J. G., 2006. Transcriptional regulation of Dictyostelium pattern formation. EMBO Rep. 7, 694-698.
Williams, J. G., 2010. Dictyostelium finds new roles to model. Genetics. 185, 717-26.

Table I. Production of spores by wild type and mef2Λ-mutant strains

Strain	Wild type	*mef2A*$^-$
AX4	100 ± 13,5	61,16 ± 12,5
AX2	100 ± 13,9	41,83 ± 15,81

Table II. Percentage of cells expressing prestalk (*ecmA*, *ecmB*) or prespore (*psaA*) specific promoters in Wild type (AX4) and mef2A- mutant (*mef2A*$^-$) structures.

Reporter gene	AX4	*mef2A*$^-$
ecmA::lacZ	4,79 ± 1,81	16,16 ± 5,91
ecmB::lacZ	14,09 ± 2,86	26,45 ± 5,17
psaA::lacZ	24,66 ± 8,13	8,13 ± 2,03

Table III. Spores production in wild-type/mef2A-mutant chimeric structures

	AX4	AX2
WT^{FL}/WT	42,42 ± 6,50	54,87 ± 6,79
WT^{FL}/*mef2A*$^-$	91,47 ± 11,82	94,42 ± 8,97
mef2A^{-FL}/WT	7,83 ± 2,35	5,27 ± 2,09
mef2A ^{FL}/*mef2A*	42,56 ±7,73	41,67 ± 5,63

Table IV. Genes that showed a significant difference in their expression between wild-type and *mef2A*-mutant structures developed for 16 hours, as determined by mRNA sequencing

	Increased expression in the mutant	Decreased expression in the mutant
hssA-related genes (prestalk-specific)	G0267936. -93,00 (93-1) G0268400. -80,00 (160-2) G0277741. -55,60 (1724-31) G0281001. -31,20 (780-25) G0281189 -134,75 (539-4) G0281191. 0,00 (79-0) G0281195. -35,33 (106-3) G0281197. -55,50 (111-2) G0282307. -106,67 (320-3) G0283713. -72,75 (291-4) G0293356. -11,24 (281-25) hssA. -10,00 (1550-155)	G0293362. 10,74 (26-279) G0281013. 11,95 (19-227)
Small proteins (57-59 aa). (prestalk specific)	G0283421. -34,33 (515-15) G0283465. -118,67 (356-3) G0283501. -25,19 (3929-156) G0283503. -41,42 (994-24) G0283505. -33,95 (1935-57) G0283507. -38,25 (1224-32) G0283511. -9,55 (2015-211) G0283515. -14,28 (2313-162) G0283519. -55,88 (950-17) G0272188 -15,28 (2119-139) G0269674 - 15,57 (794-51) G0284283 (111-0) G0283395 (97-0) G0271888 - 40,50 (81-2) G0269672 - 7,46 (574-77)	
Small proteins (69-72 aa) (prespore specific)		G0285863. 28,07 (208-7523) G0284623. 35,48 (29-1029) G0271110 13,91 (11-153)
Tiger-family proteins	tgrF1. -39,85 (518-13)	tgrC5. 18,90 (10-189)
polyketide synthase familiy	Pks32.. -6,62 (1205-182)	
Transcription regulation	mybC. -10,86 (228-21)	comH. 11,53 (184-2123) G0288967. 17,07 (96-1639) G0290847. 21,74 (80-1739) G0290855 32,39 (41-1328) G0271438. 65,50 (4-262) **srfC**. 0,00 (0-73)
Developmental genes	St15. -7,69 (1084-171) psiN. -41,51 (5645-136)	psiI; 26,06 (469-12220)
Signaling proteins	Omt12. -19,79 (277-14) arrK. -10,65 (213-20)	hspC. 15,69 (26-408)
Metabolism	osbH; -38,33 (115-3) G0278647 - 23,43 (164-7)	fhbB; 6,54 (525-3433)
Putative Membrane proteins	G0275535. -6,03 (3197-530) G0289143 - 9,79 (1116-114) G0287195 -10,05 (774-77) G0284683 - 11,87 (273-23)	G0285697. 451,2 (5-2256) G0267564 16,32 (41-669) G0272714 10,99 (73-802) G0270342 10,81 (1679-18158) G0272042 8,53 (459-3915)
Translation regulation		Rpl32. 79,86 (7-559) R52. 10,44 (195-2035)
Other functions	G0277795. -23,55 (259.11)	G0284969. 12,87 (93-1197) cog2. 12,41 (539-6687) G0276325. 32,62 (8-261) G0290965. 12,31 (13-160)

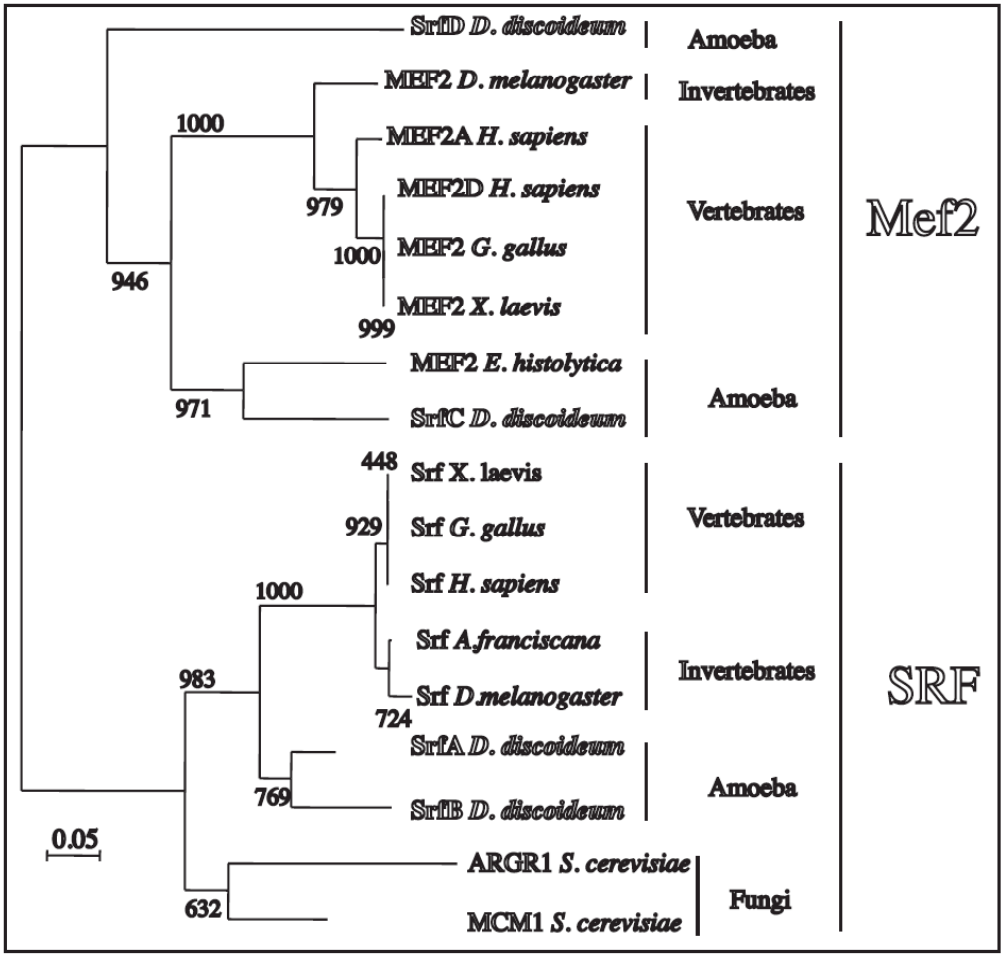


Figure 1

Figure 1. Phylogenetic tree of the *D. discoideum* MADS-box-containing proteins.

The amino acid sequences of the MADS-box region from MEF2 and SRF proteins from vertebrates (H. sapiens, X. laevis, G. gallus), invertebrate (D. melanogaster, A. franciscana) animals, fungi (S. cerevisiae) and amoeba (E. histolytica) were compared to those of the four *D. discoideum* MADS-box containing proteins (SrfA, B, C and D) using the Clustal W program at the online Biology Work Bench facility from the San Diego Supercomputer Center (http://workbench.sdsc.edu). Phylogenetic trees were determined using the neighbor-joining method and the Clustal X program. A random generator seed of 111 was used and 1000 bootstrap trial were calculated and the number of times that each branch was obtained is indicated at the base of each branch. The tree was drawn using the njplot program. The evolutionary distance scale, calculated as a fraction of amino acid changes, is indicated at the left lower corner of the figure.

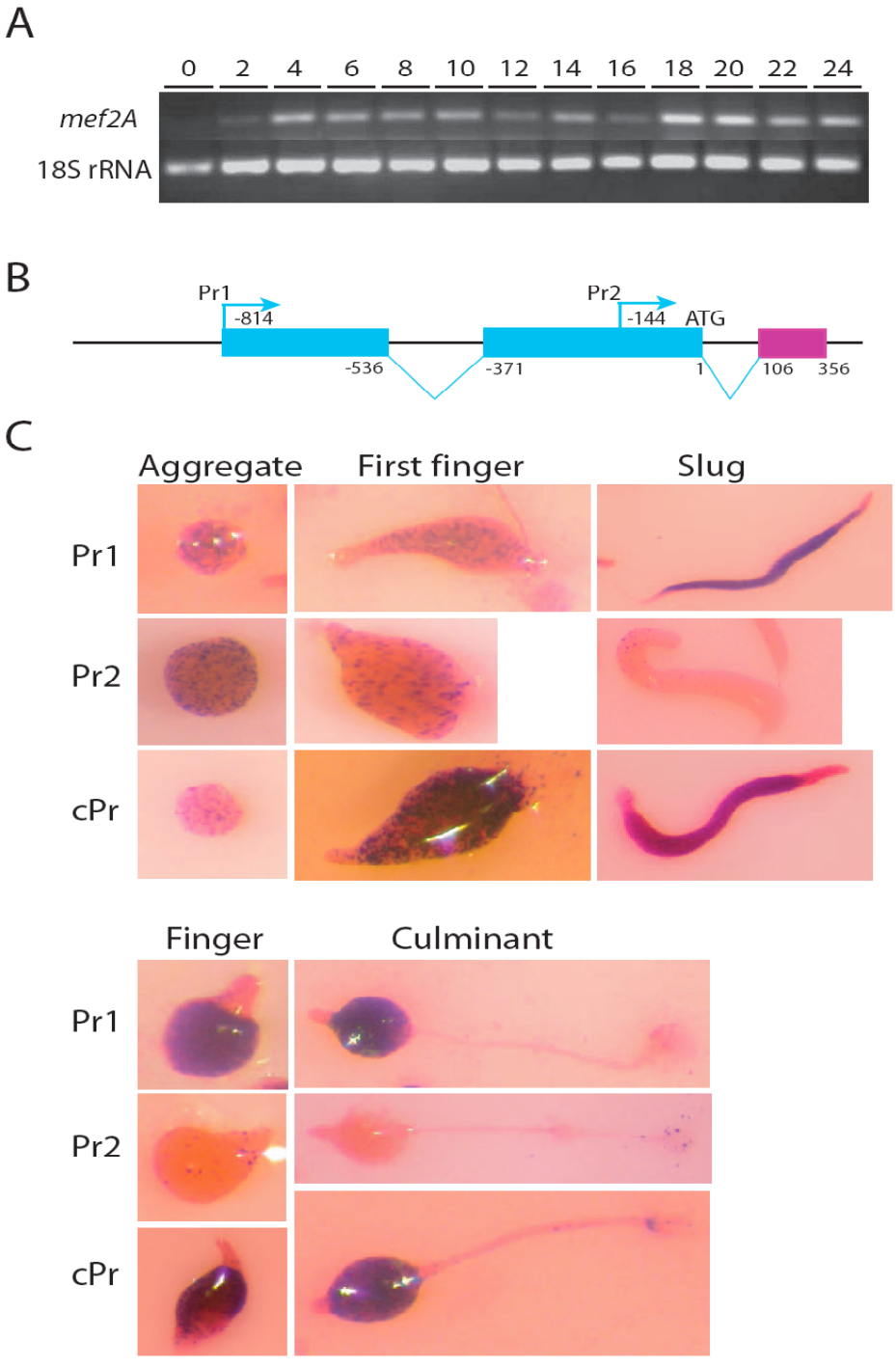

A
0 2 4 6 8 10 12 14 16 18 20 22 24
mef2A
18S rRNA
B
Pr1
-814
Pr2
-144
ATG
-536
-371
1
106
356
C
Aggregate
First finger
Slug
Pr1
Pr2
cPr
Finger
Culminant
Pr1
Pr2
cPr

Figure 2

Figure 2. Structure and expression of the *mef2A* gene.

Panel A. RNAs were isolated from AX4 cells at growth (0) or after the indicated hours of development on Nitrocellulose filters (2-24). Purified RNAs were converted into cDNA and the relative expression of *mef2A* or the large mitochondrial rRNA, used as internal control, were determined by PCR reactions.

Panel B. RNA purified from AX4 cells developed for 8 hours en Nitrocellulose filters was converted into cDNA and the 5' end extended using the MD3-11 oligonulceotide. Two different amplification products were obtained, cloned in pGEMT and 10 independent colonies sequenced. Nucleotide sequences were aligned to the genomic DNA sequence to identify exon sequences and to determine transcription initiation sites. A diagram of the deduced structure of the gene is shown. The sequence has been numbered from the A of the translation initiation codon. Exon regions are indicated as boxes, open boxes if they code for untranslated regions and filled boxes if they code for translated regions. Transcription initiation sites are indicated by arrows. The location of the intron/exon borders is indicated on the lower part of the diagram and that of the transcription initiation sites in the upper part.

Panel C. The activity of the *mef2A* promoter regions was studied using lacZ reporter vectors. The region from the 3' end of the closest upstream gene (dstB) to transcription initiation site 1 (Pr1) and from the end of exon 1 to the transcription initiation site 2 (Pr2), or the complete promoter region (cPr) were cloned in reporter vectors driving the expression of a lacZ gene coding for a short-lived form of _-galactosidase . The vectors were transfected into AX4 cells and lacZ expression analyzed at different developmental stages (Aggregate, First finger, Slug, Finger and Culminant) by hystochemistry, using the Xgal substrate. Pictures were taken using a Leica stereomicroscope.

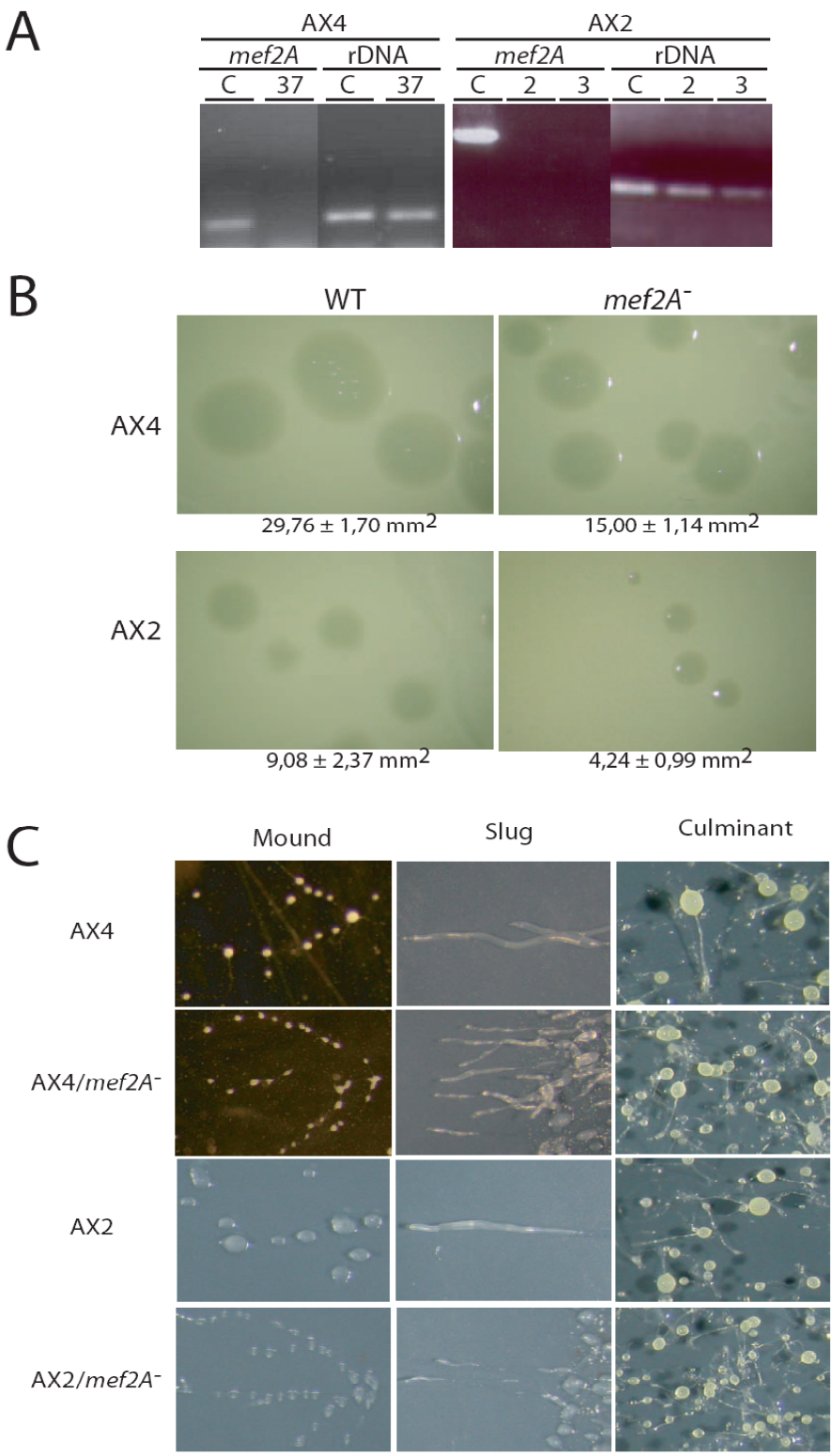

A
AX4
AX2
mef2A
rDNA
mef2A
rDNA
C 37 C 37
C 2 3 C 2 3
B
WT
mef2A-
AX4
29,76 ± 1,70 mm2
15,00 ± 1,14 mm2
AX2
9,08 ± 2,37 mm2
4,24 ± 0,99 mm2
C
Mound
Slug
Culminant
AX4
AX4/mef2A-
AX2
AX2/mef2A-

Figure 3

Figure 3. Generation and analyses of *mef2A*-mutant strains.

Panel A. Mutant AX2 and AX4 strains were generated partially deleting the *mef2A* gene by homologous recombination. DNA was isolated from several clones and analyzed by PCR using oligonucleotides specific for the *mef2A*-deleted region (*mef2A*) or for ribosomal DNA (rDNA), used as internal control. The results obtained from non-mutated (AX4, AX2) and mutated (clones 37, 2 and 3) are shown.

Panel B. Wild-type cells (AX4, AX2) and *mef2A*-mutant cells (*mef2A*-), derived from AX4 or AX2 cells, were grown on *K. aerogens* for 4 days. Pictures of the colonies formed were taken and their size determined in three independent experiments. The average area of the colonies and the standard deviations are indicated under each picture.

Panel C. Wild-type (AX4, AX2) and mutant cells (AX4/*mef2A*-, AX2/*mef2A*-) were collected and cultured under starvation conditions to study multi-cellular development. The initial steps of aggregation, streaming and mound formation were assayed under submerged conditions (Mound column). For later stages of slug (slug) and fruiting body (Culminant) formation cell were lied on Nitrocellulose filters. Pictures were taken using a Leica stereomicroscope.

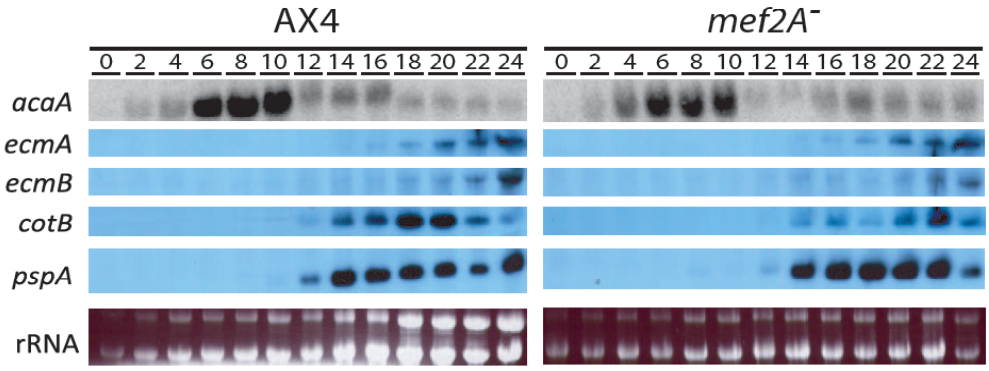


Figure 4

Figure 4. Expression of developmental genes in *mef2A*-mutant strains.

RNA was isolated from wild-type (AX4) or *mef2A*- mutant (*mef2A*-) growing cells (time 0) or structures developed on Nitrocellulose filters for 2 to 24 hours (times 2-24). Expression of acaA (adenylyl ciclase A), ecmA (extracellular matrix A), ecmB (extracellular matrix B), cotB (spore coat protein B) and pspA (prespore A) genes was determined by Northern blots. The acaA gene is expressed at maximal levels during aggregation, ecmA and ecmB genes are expressed in prestalk cells while cotB and pspA are expressed in prespore cells. The electrophoretic migration of the ribosomal RNAs is shown in the lower panel as a sample loading control.

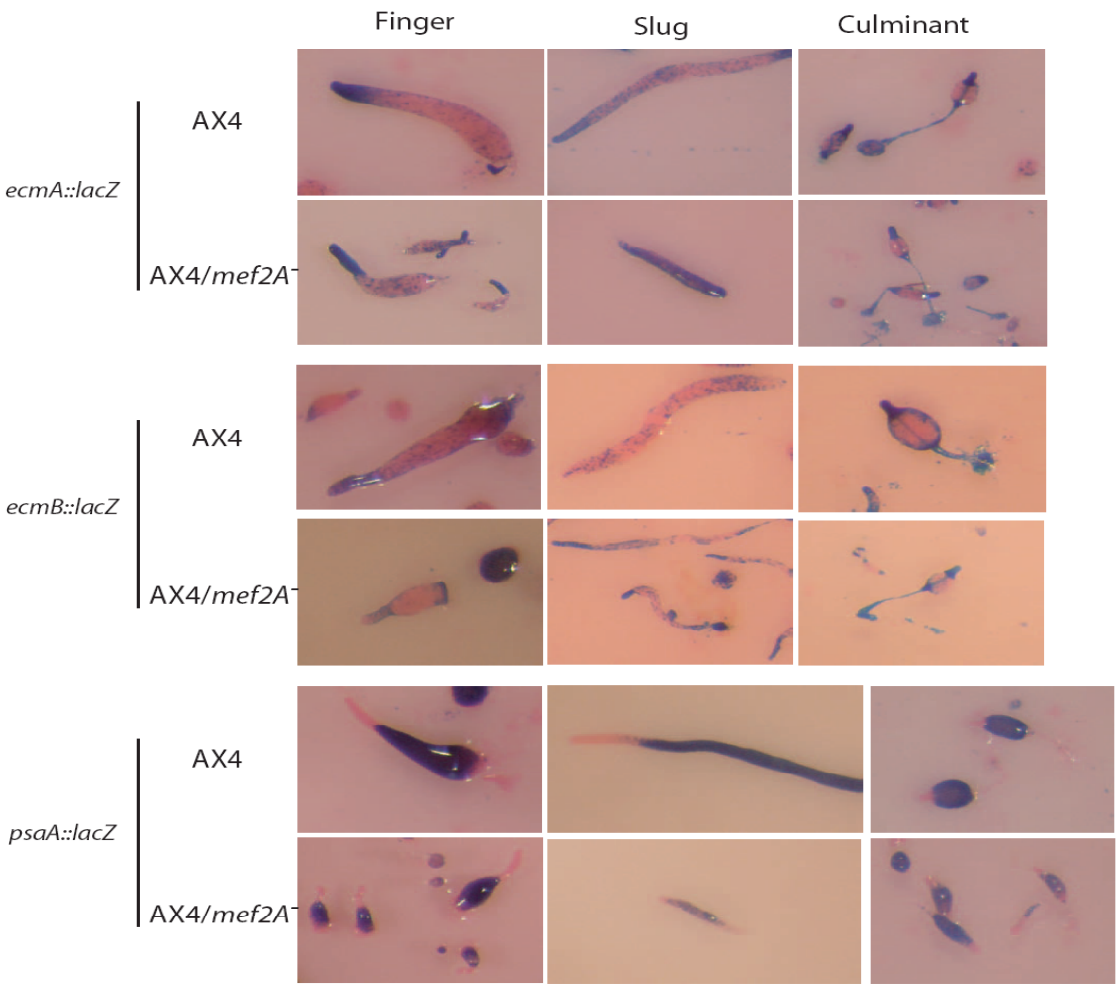


Figure 5

Figure 5. Distribution of prestalk and prespore cells in *mef2A*-mutant developmental structures.

Wild-type cells (AX4) and *mef2A*-mutant cells (*mef2A*-) were transfected with reporter vectors where lacZ was expressed under the control of the promoter region from the prestalk-specific genes ecmA (ecmA::lacZ) and ecmB (ecmB::lacZ) or the prespore-specific gene psaA (psaA::lacZ). Transfected cells were lied on Nitrocellulose filters to induced multi-cellular development. Structures were collected at the finger, slug and culminant stages of development and lacZ expression determined by hystochemical Xgal staining. Pictures were taken using a Leica stereomicroscope, after counter-staining with eosine.

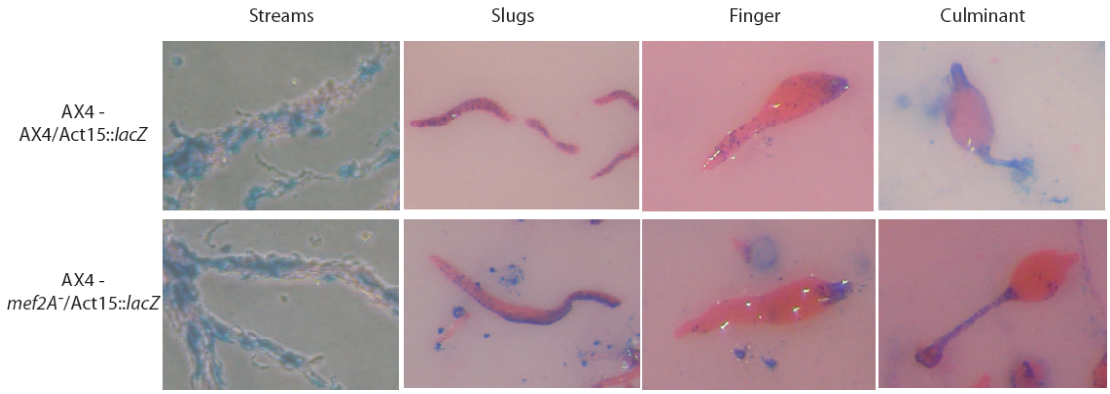


Figure 6

Figure 6. Development of *mef2A*-mutant cells in combination with wild-type cells.

Wild-type, AX4, cells were mixed in a 4:1 proportion with either wild-type or *mef2A*-mutant cells transfected with a Act15::lacZ reporter gene (AX4/Act15::lacZ; *mef2A*-/Act15::lacZ). Cell mixtures were allowed to develop under submerged conditions (streams) or on Nitrocellulose filters (slug, finger, culminant). Structures were collected at the indicated developmental stages and lacZ expression determined by Xgal hydrolysis. Structures were stained with eosine and observed under a Leica stereomicroscope.

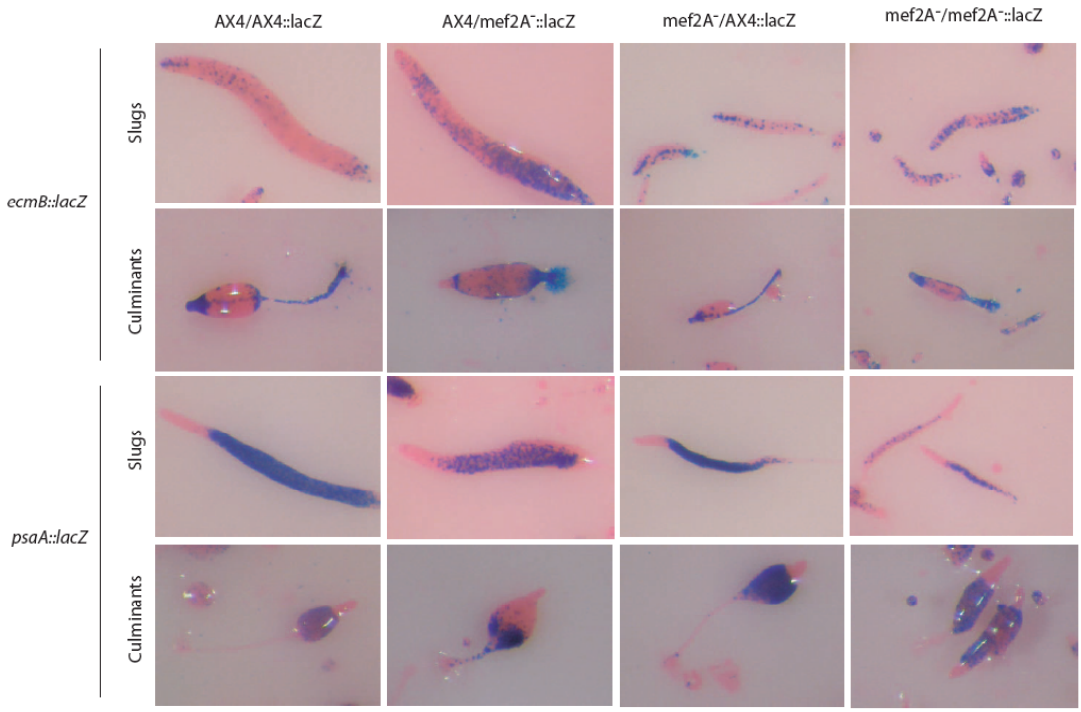


Figure 7

Figure 7. Distribution of *mef2A*-mutant cells, expressing prestalk or prespore markers, in developmental structures formed in combination with wild-type cells.

Wild-type (AX4) or *mef2A*-mutant (*mef2A*-) cells were mixed in a 4:1 proportion with cells transfected with reporter vectors (AX4-lacZ, *mef2A*-lacZ) expressing lacZ under the control of the prestalk-specific ecmB promoter (ecmB::lacZ) or the prespore-specific psaA promoter (psaA::lacZ). Cell mixtures were allowed to develop on Nitrocellulose filters and slug of culminant structures were collected and lacZ expression determined by Xgal hydrolysis. The two upper rows of pictures show the distribution of cells expressing the prestalk ecmB promoter and the two lower rows those expressing the psaA prespore promoter. Structures were stained with eosine and observed under a stereomicroscope.

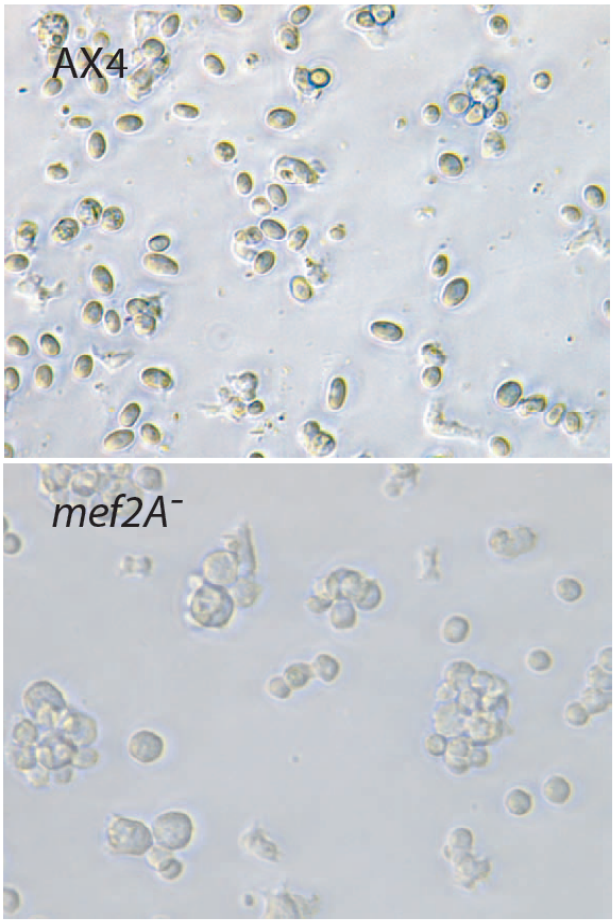


Figure 8

Figure 8. In vitro differentiation of wild-type and *mef2A*-mutant cells into spores.

Wild-type (AX4) and *mef2A*-mutant (*mef2A*-) cells were incubated in the presence of Br-cAMP, a cell-permeable derivative of cAMP, to induce spore differentiation. Pictures were taken after 30 hours of incubation using a TS100 Eclipse Nikon microscope.

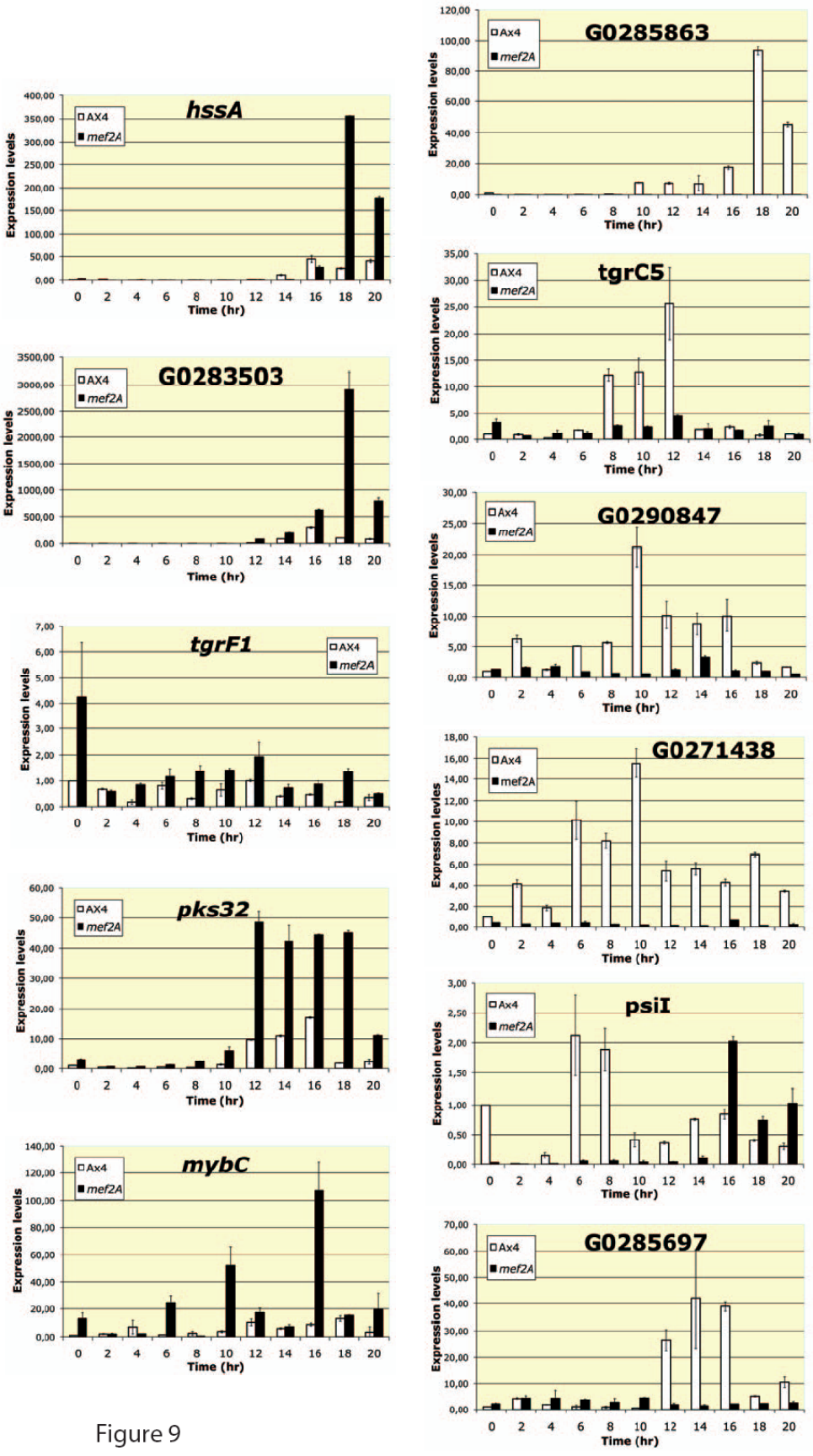

hssA
AX4
mef2A
Expression levels
Time (hr)
G0285863
Ax4
mef2A
Expression levels
Time (hr)
G0283503
AX4
mef2A
Expression levels
Time (hr)
tgrC5
AX4
mef2A
Expression levels
Time (hr)
tgrF1
AX4
mef2A
Expression levels
Time (hr)
G0290847
Ax4
mef2A
Expression levels
Time (hr)
pks32
AX4
mef2A
Expression levels
Time (hr)
G0271438
Ax4
mef2A
Expression levles
Time (hr)
mybC
Ax4
mef2A
Expression levels
Time (hr)
psiI
Ax4
mef2A
Expression levels
Time (hr)
G0285697
Ax4
mef2A
Expression levels
Time (hr)

Figure 9

Figure 9. Developmental pattern of expression of representative genes differentially regulated in Wild-type and *mef2A*-mutant structures, as determined by mRNA sequencing.

RNA was isolated from wild-type (AX4) or *mef2A*-mutant (*mef2A*) cells at growth (time 0)or from structures developed for the indicated times on Nitrocellulose filters (time 2-20). RNAs were converted into cDNA and the level of expression of each gene determined by quantitative PCR. A fragment of the large mitochondrial ribosomal RNA was used as internal control for expression quantification. The relative value of 1 was assigned to the expression level of growing wild-type cells for each gene. Open bars correspond to wild-type expression levels and black bars to *mef2A*-mutant expression levels. Panels on the left correspond to genes that were expressed at highest levels in *mef2A*-mutant structures, according to the mRNA sequencing analyses, and those on the right to genes that were expressed at higher levels in wild-type cells.

Discusión

DISCUSIÓN

Los factores de transcripción de la familia MADS-box son proteínas implicadas en importantes procesos biológicos. Estas proteínas contienen un dominio altamente conservado de unión a ADN y dimerización llamado caja MADS. Se han identificado dos tipos distintos de proteínas que contienen caja MADS, los similares a SRF (tipo I) y los similares a MEF2 (tipo II). Ambos grupos se pueden encontrar en animales, plantas y hongos. Los miembros de la familia MADS generalmente reconocen una secuencia rica en Adeninas y Timinas localizada en las regiones reguladoras de los genes cuya expresión está controlada por esta familia de factores. Las proteínas de la familia MADS-box son factores de transcripción con capacidad para interaccionar con una variedad muy amplia de cofactores y por tanto, son capaces de regular la expresión de múltiples genes y procesos (92).

SRF, en mamíferos, desempeña funciones tan importantes como por ejemplo el control de la diferenciación y de la proliferación del tejido muscular. Además, mediante estudios en células madre (células ES) se ha implicado a SRF en procesos de migración, adhesión y extensión o *"spreading"* de las células, todas ellas funciones relacionadas con el citoesqueleto de actina. Muchos genes regulados directamente por SRF son genes con funciones en el citoesqueleto. Por otro lado, varios estudios muestran una cierta correlación entre el cáncer humano y niveles altos de SRF o de SRF unido a secuencias CArG (18).

En otros organismos como *Saccharomyces cerevisiae* existen dos proteínas homólogas a SRF, Mcm1 y Arg80. Mcm1 está implicado en el metabolismo de la Arginina, al igual que Arg 80, pero presenta además, otros papeles pleiotrópicos en la célula tales como el control de la transcripción dependiente de ciclo celular en las transiciones M/G1 y G2/M, el control del apareamiento (*mating)*, etc. En *Drosophila melanogaster*, DSRF está implicado en el desarrollo del sistema traqueal, que participa en el intercambio de gases, y en el desarrollo de las alas.

Como hemos comentado anteriormente, MEF2 pertenece a la familia de factores de transcripción que poseen caja MADS. *Saccharomyces cerevisiae*, *Drosophila melanogaster* y *Caenorhabditis elegans* tienen un único gen homólogo a *Mef2*, mientras que en vertebrados hay cuatro, *Mef2a, b, c* y *d*. Rlm1, el homólogo de MEF2 en levadura funciona como un efector de la vía de las MAP quinasas, regulando una gran cantidad de genes implicados en la biosíntesis de la pared celular. En *D. melanogaster*, *mef2* se expresa en mesodermo y posteriormente en diversos linajes musculares, donde está implicado en diferenciación de mioblastos. Por otro lado, los cuatro genes de vertebrados presentan patrones de expresión espacial y temporal distintos pero solapantes en tejidos embrionarios y adultos, siendo especialmente mayoritarios

en músculo estriado y cerebro. Además, en vertebrados, Mef2 es también expresado en linfocitos, cresta neural, músculo liso, endotelio y hueso, incluso en algunos estudios lo presentan como ubicuo. En tejidos adultos, las proteínas Mef2 representan un punto clave en la respuesta a stress, programas de remodelación, supervivencia celular, apoptosis y proliferación. En cada una de estas funciones, los genes diana dependen de modificaciones post-transcripcionales específicas de Mef2 y de interacciones con cofactores (111).

La posibilidad de una conservación evolutiva en las funciones de los factores de transcripción de la familia MADS-box, nos llevó a la búsqueda de posibles proteínas homólogas en el sistema modelo de *Dictyostelium discoideum*. Este es un sistema sencillo reconocido como modelo experimental debido a la multitud de ventajas que tiene para su uso en el laboratorio. Entre dichas ventajas se encuentra su parecido a las células animales en muchos aspectos estructurales y funcionales, y la posible adaptación y aplicación de técnicas moleculares de manera más sencilla, como se explicó en la Introducción.

La secuenciación del genoma completo de *Dictyostelium discoideum* (35) permitió la identificación de cuatro genes que codifican proteínas con una región similar al dominio MADS-box, *srfA, srfB, srfC y srfD*. Dos de ellos, *srfA* y *srfB* (Capítulo1), codifican proteínas similares a SRF, mientras que los otros dos, *srfC* y *srfD*, son más similares a MEF2 (Capítulo 4).

Los tres genes estudiados hasta el momento, ***srfA, srfB y mef2A*** (***srfC***) están implicados de alguna manera en la diferenciación de distintos tipos celulares. Los fenotipos de las cepas mutantes para estos genes son bastante heterogéneos y diferentes entre sí. El gen *srfA*, estudiado anteriormente, está implicado en la diferenciación de las células pre-tallo de las regiones pstA y pstO, presentando un fenotipo claro en la morfología y migración de los *"slugs"* además de no producir esporas correctamente diferenciadas. *SrfB*, por su parte, está implicado en la diferenciación de las células de la región pstAB (células de la región organizadora del *"tip"*) y como tal presenta un fenotipo claro de retraso en la culminación. Por otro lado, *mef2A* regula la diferenciación de las células pre-espora, ya desde fases tempranas del desarrollo. El fenotipo que presenta es de alteración en la proporción de los tipos celulares y de reducción en la producción de esporas. Estudiando la estructura de estos tres genes (*srfA, srfB y mef2A*) también encontramos algo en común. Su expresión está controlada por diferentes regiones reguladoras que hacen que se puedan expresar en estadios del desarrollo distintos y en tipos celulares específicos. Esta característica es común a otros genes de *D. discoideum* relacionados con la regulación del desarrollo. Entre ellos está *pdsA,* que codifica una Fosfodiesterasa extracelular (40), *carA* (75) que codifica un receptor de AMPc y *acaA* (Capítulo 2) (45) que codifica a la

Adenilato ciclasa implicada en este proceso. Es posible que la existencia de promotores alternativos en todos estos genes sea una adaptación evolutiva para regular la participación de una proteína en diferentes condiciones de crecimiento o en diferentes procesos.

Al menos en dos de los casos, *srfB* y *mef2A*, alguno de los promotores alternativos dirige la expresión durante la fase vegetativa por lo que las cepas mutantes presentan fenotipos, también, durante el crecimiento. En el caso de la cepa mutante para *srfB* (***srfB***$^{-}$), las células en crecimiento, presentan deficiencias en macropinocitosis, citocinesis, migración y claramente, en la transición de la fase crecimiento a la de desarrollo pluricelular. Varios de estos fenotipos tienen cierta relación con posibles defectos en el funcionamiento del citoesqueleto de actina. Como mencionamos anteriormente, SRF regula, tanto directa como indirectamente, la expresión de muchos componentes del citoesqueleto de actina de animales, incluyendo la actina y diferentes isoformas de la miosina (95). En el estudio de expresión génica (*"arrays"*) de la cepa mutante para *srfB* no se encontraron cambios en la expresión ni de la actina ni de la mayoría de isoformas de la miosina. Sin embargo, myoI (proteína relacionada con el citoesqueleto), dos de las cadenas de la miosina, ELC y RLC y dos proteínas reguladoras (Ponticulina y Cofilina) si presentaban cambios. Hemos de mencionar que la regulación de la expresión de las actinas y las miosinas en *D. discoideum* es muy compleja ya que hay más de 30 genes que codifican actinas (35), 13 que codifican la cadena pesada y de 4 a 6 para la cadena ligera de la miosina (66). El proceso de proliferación en las cepas mutantes de *srfA* (***srfA***$^{-}$), parece correcto; sin embargo, no hemos sido capaces de obtener cepas mutantes para *srfA y srfB*. Como hemos mencionado anteriormente, las mutaciones individuales de ambos genes no producen defectos graves en proliferación, lo que nos podría llevar a pensar en la existencia de cierta redundancia funcional, de forma que *srfA* complementa la función de *srfB* durante la proliferación celular y viceversa. En el caso de la cepa mutante para *mef2A* (***mef2A***$^{-}$) se ha observado menor crecimiento en bacterias, aunque este hecho no parece deberse a un defecto en la fagocitosis (datos no mostrados) no ha sido caracterizado a fondo aún. No obstante, *mef2A* ha sido identificado como uno de los genes cuya expresión se ve regulada dependiendo del substrato de crecimiento (bacterias o medio axénico) (120).

Otra característica común que hemos encontrado para *srfA, srfB* y *mef2A* es la dificultad para encontrar sitios de unión consenso en los promotores de los genes cuya expresión está potencialmente regulada por estos factores. En el caso de los posibles genes regulados por *srfB* exceptuando el promotor de la actina 15 (*act15*) que presenta una posible región de unión consenso, no hemos sido capaces de encontrar más. Es posible que no se haya conservado dicha secuencia ($CC(A/T)_6GG$) a lo largo de la evolución de estos organismos. En el caso de mef2A, algunos de los genes que están menos expresados en el mutante de *mef2A* parecen ser

totalmente dependientes de la expresión de este factor de transcripción. Como en el caso de SrfA y B, la regulación de la expresión de estos genes puede llevarse a cabo mediante la unión directa de Mef2A a sus regiones reguladoras, o mediante la regulación por parte de Mef2A de la expresión de otros factores de transcripción que controlen la expresión de dichos genes. El sitio de unión en el ADN al que se une Mef2A se ha conservado a lo largo de la evolución ($CTA(A/T)_4ATG$). Hemos buscado dicha secuencia en las regiones promotoras de algunos de los genes dependientes de mef2A pero sólo hemos encontrado tres sitios de unión de Mef2A en el promotor del gen *tgrC5*. Por lo tanto, se requeriría de un estudio más amplio de la región que reconocen SrfA, SrfB y Mef2A y de cuál es el mecanismo por el cual regulan la transcripción de sus genes diana.

Una vez determinado el fenotipo de los mutantes, procedimos a profundizar en los procesos biológicos afectados y en el papel que juegan *srfB* y *mef2A* en el desarrollo de los mismos.

Uno de los defectos más característicos de la cepa mutante para *srfB* es que su respuesta a la escasez de alimento (ayuno) es más rápida que la de las células *"wild-type"*. La formación de agregados, sobre filtros de nitrocelulosa o sobre agar, se produce con 2 horas de antelación respecto a las células silvestres. De acuerdo con este resultado nos encontramos con que la expresión de genes tempranos como el receptor de AMPc (*carA*) o el de la Adenilato ciclasa (*acaA*) está también adelantada 2 horas aproximadamente con respecto a la de células silvestres. En concodancia, observamos que algunos de los genes que son rápidamente reprimidos tras el ayuno, como los que codifican proteínas ribosomales (1) (65) y lisosomales (17) están menos expresados en el mutante. La Discoidina, lectina importante para la adhesión celular y la correcta polarización y motilidad, está inducida prematuramente y a niveles mucho mayores en el mutante. Esta desregulación génica se recupera cuando se vuelve a expresar *srfB* en la cepa mutante. Como se sabía anteriormente, la entrada en fase de ayuno provoca en las células un cambio en el patrón de expresión génico (58) (73). Los datos obtenidos para el mutante, plantean la posibilidad de que *srfB* esté implicado en los cambios que se producen cuando las células pasan de su fase de crecimiento a su fase de desarrollo pluricelular.

En aparente contraposición con estas observaciones, las células *srfB*$^-$ muestran cierto retraso en el desarrollo cuando son incubadas en agitación. La quimiotaxis hacía AMPc, principal proceso que dirige la agregación, y la adquisición de adhesión celular resistente al tratamiento con EDTA, presentan ciertas deficiencias en las células mutantes. Sin embargo, en filtros de nitrocelulosa o agar, los agregados se forman antes que en el silvestre. Esta contradicción se podría explicar considerando que la agregación temprana, en esas condiciones,

se produce por colisiones fortuitas entre células y no por adhesiones resistente a EDTA. Apoyando esta hipótesis observamos que las adhesiones que son sensibles a EDTA son mayores en la cepa mutante, y que por ello, podría formar mayor número de grupos de células o pequeños agregados en ausencia de quimiotaxis. Además, la formación de *"streams"*, que es típico de células sometidas a quimiotaxis, no aparece cuando las células mutantes están desarrollándose en agar, pero la formación de esos pequeños agregados (no quimiotácticos) podría favorecer la consecución del desarrollo. Los estudios de quimiotaxis hacia AMPc realizados en colaboración con el Dr. Salvatore Bozzaro (Università di Torino, Italia) mostraron que la cepa mutante presentaba defectos en la migración hacia el quimioatrayente. A pesar de tener una mayor motilidad, las células *srfB*$^-$ tienen una capacidad de orientación reducida, no se polarizan y no migran, o lo hacen poco, hacia la fuente de AMPc. En su lugar, las células se mueven alrededor de su eje permaneciendo ancladas al sustrato principalmente por su parte posterior. Cuando a la cepa mutante se le suministran pulsos de AMPc se recupera el defecto en las adhesiones resistentes a EDTA, con lo que concluimos que la cepa mutante para *srfB* es capaz de responder a AMPc pero no es capaz de generar la señal de AMPc necesaria, a su vez, para expresar la glicoproteína csA y otros genes implicados en la agregación (48, 58) (89). Hay que tener en mente, sin embargo, que cuando suministramos AMPc sólo algunos de los defectos de la cepa mutante se recuperan, aumentando ligeramente la quimiotaxis.

La hipótesis de la existencia de un defecto en la señalización por AMPc nos llevó al estudio de la vía de señalización que inicia el proceso de culminación y que es desencadenada por el aumento del AMPc extracelular. El aumento del AMPc extracelular desencadena, en las células del *"tip"*, una vía de señalización que activa secuencialmente los factores de transcripción StatA y CudA, que a su vez activa la transcripción de otros muchos efectores (135). Los resultados obtenidos indican que srfB realiza una función anterior a la activación de dicha cascada de señalización. Esta hipótesis está basada en que CudA no se está expresando en la región del *"tip"* en estructuras mutantes para *srfB* y además, *srfB* se expresa correctamente en la región del *"tip"* de estructuras mutantes para los genes *statA* y *cudA*. Sin embargo, el gen *ecmB*, que también es un gen marcador de la región del *"tip"*, se expresa correctamente en la cepa mutante *srfB*$^-$. Esto nos indica que las células de la región organizadora del *"tip"* se están diferenciando al menos parcialmente, pero no a través de la vía de StatA/CudA. El gen *ecmB* también se expresa en la región del *"tip"* de mutantes para *CudA*. (44).

La molécula que inicia la culminación, el AMPc, es sintetizado por las encimas Adenilato ciclasas. Existen tres encimas de este tipo en *D. discoideum*, codificadas por tres genes, *acaA*, *acgA* y *acrA* (68). AcaA es la encima que se expresa en la región organizadora del *"tip"* y podría ser la encima responsable de la síntesis del AMPc extracelular necesario para la

iniciación de la culminación. Sabiendo todo esto, llevamos a cabo un estudio exhaustivo de la región promotora del gen de la Adenilato ciclasa (*acaA*) y de la expresión de este gen a lo largo del desarrollo de células *"wild-type"*, para luego compararlo con lo que ocurre en las células mutantes para *srfB*.

En células silvestres (Ax4), el gen *acaA* se transcribe desde tres promotores alternativos aunque la proteína que codifican todos los ARNm generados sea la misma. Los análisis funcionales indican que el promotor más distal, Promotor 1, está especialmente activo en células en agregación según se ha corroborado tanto por medición de actividad del promotor como de expresión del ARNm1. Sin embargo, el Promotor 2 es especialmente activo en la región pre-espora. El Promotor 3, es principalmente activo en células pre-tallo, pero no en todas, sólo en las células que conforman el *"tip"* o región organizadora y por último, el promotor Completo muestra un patrón de expresión que agrupa los tres patrones pero, en general, es más parecido al Promotor 3. La existencia de un promotor distal que dirige la expresión durante la agregación convertiría a *acaA* en el tercer gen implicado en la señalización mediada por AMPc que presenta semejante adaptación. En estudios previos (3) ya se había propuesto que los Dictiostélidos, para poder usar el AMPc durante la agregación como molécula quimioatrayente, habían tenido que adquirir nuevas regiones promotoras que permitieran una regulación génica más específica y controlada.

Existen dos estudios previos del promotor de *acaA*. Verkerke-van Wijk (130) y sus colaboradores caracterizaron el promotor de *acaA* aislado de un clon genómico que resultó tener una deleción interna que incluye el promotor 2 y el 3, así que la región del promotor estudiada comprende una parte del Promotor 2 (desde el nucleótido –1838 hasta el –1310) fusionado a la parte más proximal del Promotor 3 (nucleótidos –86 al –1). Este promotor es activo en células del *"mound"* y del centro organizador o *"tip"* de estructuras migratorias, con un patrón similar al presentado por el Promotor 3. Estos resultados podrían indicar la existencia de una región reguladora, que activa la expresión en células del *"tip"*, entre el nucleótido –86 y el 1, que también está presente en la región del Promotor 3 analizado. Además, Siol y sus colaboradores (121) caracterizaron la actividad promotora de un fragmento de 773 bp que corresponde a la región proximal del Promotor 3 (nucleótidos –739 al 34). Este fragmento activa la transcripción en agregados y es dependiente del factor CbfA. Estos autores demostraron que CbfA era necesario para la expresión de *acaA* durante el desarrollo (136). Ya que el Promotor 1 es específico de agregación, sería de interés determinar si este promotor, que presenta varios sitios potenciales de unión para este factor de transcripción, también depende de la activación de CbfA.

Los datos obtenidos del estudio completo de la región promotora del gen *acaA* han demostrado la expresión de este gen en varías regiones de las estructuras, lo que era previamente desconocido y abren un abanico de posibles funciones en las que podría estar implicada *acaA*. Estas nuevas funciones irían más allá de la agregación, en estadios posteriores del desarrollo tales como la regulación de la culminación o de la diferenciación de las células pre-tallo y pre-espora.

Una vez fue estudiada la actividad del promotor del gen *acaA* en células silvestres, quisimos comprobar que ocurría en las células mutantes para *srfB*. Queríamos establecer si srfB tiene algún papel regulador sobre la expresión de la Adenilato ciclasa durante el desarrollo y, en particular, durante la iniciación de la culminación. Los datos obtenidos mediante estudios histoquímicos y de RT-PCR cuantitativa indicaron diferencias apreciables en la expresión del gen *acaA* en la región organizadora del *"tip"*. Las células que expresaban *acaA* presentaban una localización menos precisa en el *"tip"* de los *"finger"* y los *"slugs"* de los mutantes de *srfB*. La actividad del Promotor 3 y el Promotor completo y del fragmento del promotor descrito por Verkerke et al. confirmaron dicho fenotipo. Estos datos podría indicarnos que el defecto en la iniciación de la culminación en el mutante de *srfB* podría ser consecuencia de que *acaA* no está correctamente expresado en la región organizadora del *"tip"*. Otra diferencia observada es que *acaA* se expresa menos en el mutante en la región pre-espora con respecto al silvestre. Esta menor expresión de *acaA* en la región pre-espora podría contribuir a una menor producción de AMPc. Este AMPc podría ser capaz de difundir hacia la región organizadora del *"tip"* contribuyendo a la inducción de la culminación. Lo sorprendente de esta observación es que, *srfB* no se expresa a niveles detectables en la región pre-espora ni en los *"fingers"* ni en los *"slugs"* durante el desarrollo (Capítulo 1) (46). Inicialmente pensamos que estos resultados podrían deberse a que las células del *"tip"* segregaran alguna molécula inductora de la expresión de *acaA* en las células pre-espora y que esta señal podía estar ausente en las células *srfB*$^-$. Para comprobar esta hipótesis realizamos experimentos de mezclas de células silvestres (Ax4) y *srfB*$^-$, portando una de las cepas un vector reportero en el que *lacZ* estaba bajo el control del Promotor 2 del gen *acaA*. Los resultados obtenidos indicaron que la presencia de células Ax4 no inducía la actividad del Promotor 2 del gen *acaA* en las células *srfB*$^-$ como predecía nuestra hipótesis inicial. Por el contrario, observamos que la presencia de células *srfB*$^-$ reprimía la actividad del Promotor 2 del gen *acaA* en las células silvestres. A la vista de estos resultados, nuestro modelo actual consistiría en que, en presencia de *srfB* en la región del *"tip"*, ciertas señales moleculares estarían reprimidas y por tanto, *acaA* se estaría expresando en la región pre-espora. Sin embargo, cuando *srfB* no se expresa, esas señales moleculares se encuentran activas reprimiendo la expresión de *acaA* en las células pre-esporas. Es posible que esas señales represoras se activen desde el principio del desarrollo pero en presencia de srfB se vean

silenciadas en la región del *"tip"* al comienzo de la culminación. La existencia de señales intercelulares que se producen en un tipo celular pero actúan sobre otro es bien conocida en *D. discoideum*. Por ejemplo, en la diferenciación de las células pre-espora y pre-tallo, DIF-1 juega un papel clave, se produce en las células pre-espora pero activa la diferenciación de las células pre-tallo (9). Sin embargo, pensamos que esta sería la primera vez que se describe un mecanismo de señalización con origen en las células pre-tallo que regula la expresión génica en las células pre-espora. Con este modelo en mente, planteamos que srfB podría tener una función activadora sobre la expresión de *acaA* en la región pre-espora y que esa expresión sería necesaria para la progresión del desarrollo, incluyendo el inicio de la culminación.

Como hemos mencionado anteriormente el estudio de la función biológica de *mef2A* se llevó a cabo mediante la generación de cepas mutantes, tanto en la cepa *"wild type"* Ax4 como en Ax2 con fenotipos similares. Hay que destacar que la sobre-expresión de Mef2A, tanto bajo el control del promotor de Actina15 como de su propio promotor, produjo un fenotipo similar al de las cepas mutantes lo que impidió realizar estudios de complementación de las cepas mutantes. Los resultados obtenidos indicaban que ***srfC* (*mef2A*)** estaba implicado en la determinación y en la diferenciación de las células pre-espora y un grupo de células pre-tallo en *D. discoideum*. Este papel de *mef2A* en la determinación y diferenciación de las esporas se fundamenta en varias evidencias. El gen *mef2A,* para empezar, se expresa en la región pre-espora desde las fases más tempranas del desarrollo, ya que su expresión se induce a las 4 horas. Los genes marcadores de la región pre-espora se expresan menos en las células mutantes para *mef2A*, como puede observarse en los análisis de expresión (*Northern Blot*) y en las histoquímicas realizadas con vectores que expresan el gen *lacZ* bajo los promotores de estos genes. El defecto observado en la expresión de los genes marcadores de células pre-espora es un defecto considerado autónomo celular es decir, aún en presencia de células "*wild-type"* y de las señales que ellas producen, las células mutantes no se diferencian a células pre-espora, en experimentos de mezclas de ambas cepas. Por otro lado, los genes marcadores de células pre-tallo están sobre-expresados. Además, las células mutantes para *mef2A* no se diferencian *in vitro* a esporas (experimentos realizados en colaboración con la Dra. Teresa Suarez, CIB, España), e *in vivo*, producen la mitad de esporas que una cepa "*wild-type"*

El análisis de la expresión génica mediante secuenciación masiva corrobora nuestra hipótesis. Muchos de los genes cuya expresión desciende en los mutantes de *mef2A* se expresan específicamente en células pre-espora. Algunos de los cuales son, *psiI ("prespore inducing factor"),* el grupo de proteínas pequeñas específicas de pre-espora, tgrC5, un grupo de proteínas posiblemente implicadas en regulación transcripcional y proteínas de membrana mostradas en la Tabla IV del Capítulo 4. También se han observado diferencias entre las células mutantes y las

"wild-type" en la expresión de algunos de los genes usados como marcadores de células pre-espora, aunque no pasaron los requerimientos impuestos para el análisis de los datos de secuenciación masiva de ARNm (más de tres veces de diferencia en los niveles de expresión y un valor $p < 0,01$). Por ejemplo, *cotA* se expresaba 2,12 veces más en Ax4 que el mutante, *cotC* 2,02 veces, *pspD* 1,95 veces y *pspB* 2,52 veces más.

En la misma línea de nuestra hipótesis se observó que la mayoría de genes que estaban sobre-expresados en la cepa mutante eran específicos de células pre-tallo. Algunos de ellos, genes relacionados con *hssA* o con genes que codifican proteínas pequeñas como puede verse en la Tabla IV del Capítulo 4. Dos de los genes relacionados con *hssA* mostraban mayor expresión en *"wild-type"* con respecto al mutante en los análisis de secuencias mientras que, en los análisis por RT-PCR cuantitativa aparecían aumentados en la cepa mutante en fases del desarrollo posteriores al tiempo (16 horas) que se usó para el estudio masivo de secuencias (datos no mostrados). El gen *ecmA,* que es uno de los marcadores de células pre-tallo, está expresado 1,5 veces más en la cepa mutante de acuerdo con los datos de la secuenciación masiva, mientras que *ecmB* está expresado 1,5 veces más en la cepa *"wild-type"* en el mismo tiempo de desarrollo.

Mef2A parece estar implicado también en la determinación y diferenciación de la sub-población de células pre-tallo localizada en la parte superior de los *"mound" y "fingers"* y en la parte anterior de los *"slugs"*. Estas células se caracterizan por la expresión de *ecmB* e incluyen las células organizadoras del *"tip"* que regulan el proceso de culminación (135). Las células mutantes que expresan un vector donde la expresión de *lacZ* está bajo la dirección del promotor de *ecmB* (ecmB::*lacZ*) no participan en la formación del *"tip"* en los experimentos de mezcla con células silvestres en desarrollo (Fig. 6,7 del Capítulo 4). Aunque no lo mostramos, las células mutantes para *mef2A* no diferencian correctamente *in vitro* a células pre-tallo (que expresen *ecmB*). Por un lado, no se ha detectado expresión de *mef2A* en células pstAB, pero es posible que el papel que lleva a cabo este factor en la diferenciación, venga ya determinado desde la fase de *"mound",* donde se expresa en la mayor parte de las células, bajo la dirección de dos promotores alternativos. También cabe la posibilidad de que el número de células que expresan *mef2A* en la región PstAB sea demasiado bajo como para ser detectado mediante ensayos con vectores reporteros.

La existencia de diferencias en la especificación o diferenciación de los tipos celulares podría explicar los fenotipos observados en la cepa mutante para *mef2A*. Por ejemplo, la existencia de un mayor número de células pre-tallo que se adhieren más entre ellas podría provocar la ruptura de los *"streams"*. Además, algunos de los genes que se encuentran

desregulados en los mutantes, como son los de la familia tgr (*tgrF1 y tgrC5*) está implicada en adhesión (8). Sin embargo, no se han detectado diferencias en la adhesión celular en presencia de calcio entre las células silvestres y las *mef2A*$^-$ (datos no mostrados). Los *"slugs"* presentan una distribución de las células pre-tallo y pre-espora anómala (Fig5 Capítulo 4), lo cual podría explicar los defectos en su tamaño y motilidad. Además, la región organizadora del *"tip"* se encuentra alterada también en los *"slugs"* como puede observarse por la expresión del gen *ecmB*. Esta región juega un papel regulador en la migración de los *"slugs"*. Cuando las células mutantes son cultivadas en condiciones que favorecen la formación de los *"slugs"*, se forman una mayor cantidad de *"mounds"* pequeños con bastantes células pre-tallo localizadas en la región basal y que parece que impiden la formación de los *"slugs*". A pesar de los defectos comentados, también queda claro que *mef2A* no es absolutamente necesario para el desarrollo de *D. discoideum* ya que, a pesar de todo, se forman un gran número de cuerpos fructíferos y se forman esporas aunque en menor número. Sin embargo, nos gustaría proponer que *mef2A* participa en una red de factores de transcripción que regula la diferenciación celular y que podrían compensar parcialmente la ausencia de *mef2A*. Uno de ellos podría ser SrfD, que también es homólogo al factor de transcripción Mef2 y cuya función aún no ha sido estudiada. Además de srfD, hay evidencias de la implicación de otros reguladores transcripcionales en el proceso de diferenciación de las células pre-espora. Se ha descrito que la mutación de las deacetilasas de histonas (115) y de proteínas de unión a cromatina (33) afecta a la diferenciación de células pre-espora y a la distribución y proporción de cada tipo celular. Además, la expresión de otros genes que codifican reguladores transcripcionales es dependiente de *mef2A*. Un ejemplo sería *comH*, que codifica un factor de transcripción de unión a GATA, que se expresa en células pre-espora. G0288967 codifica un factor de transcripción con dominio-sandwich y G0290847, G0290855 y G0271438 codifican proteínas con dominios posiblemente implicados en la unión a ADN y en la regulación transcripcional. El gen homólogo a retinoblastoma, *rblA,* controla también la preferencia de las células por diferenciar a un tipo celular o a otro (78). Este gen se expresa en la región pre-espora; cuando mezclamos células wild-type con células mutantes para *rblA*, estas presentan cierta preferencia por diferenciar a células pre-tallo, como también ocurre con las células mutantes para *mef2A*. Hay que mencionar que *rblA* se induce más tarde que *mef2A* durante el desarrollo y parece tener un papel más importante en la diferenciación de las esporas.

La actividad del factor de transcripción Mef2A de vertebrados está finamente regulada por señales extracelulares; una de las más conocidas es la asociación inhibitoria de Mef2A con Deacetilasas de histonas de clase II (HDACs). Determinadas señales extracelulares producen la fosforilación de las HDACs liberando así Mef2A. Mef2A, a su vez, puede ser directamente fosforilado por proteína quinasas dependientes de mitógeno o MAP quinasas (111) regulando

así su actividad transcripcional. *D. discoideum* codifica sólo dos MAP quinasas, ErkA y ErkB. ErkB se ha descrito que está implicada en la diferenciación de esporas (100). En futuros experimentos sería interesante esclarecer sí Mef2A forma parte de la vía reguladora en la que participa ErkB y que está implicada en la diferenciación de esporas. Hemos intentado complementar la cepa mutante para *ErkB* con una proteína de fusión en la que Mef2A estaría constitutivamente activa (mef2A/VP16) pero la expresión de esta forma, en células *"wild-type"* provoca defectos en el desarrollo, lo cual invalida la aproximación experimental.

Conclusiones

CONCLUSIONES

1.- En *Dictyostelium discoideum* existen cuatro genes, *srfA, srfB srfC y srfD,* que codifican proteínas homólogas a la familia de factores de transcripción de la familia MADS-box. Dos ellos, *srfA y srfB* codifican proteínas similares a SRF, mientras que los otros dos, *srfC y srfD*, codifican proteínas más similares a Mef2.

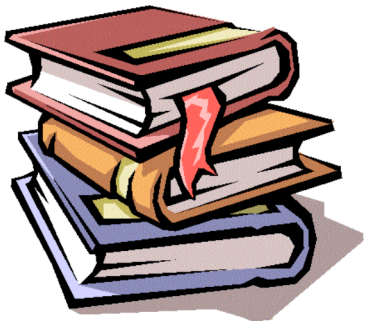
2.- Los genes *srfB* y *mef2A* (*srfC*), al igual que *srfA*, presentan promotores alternativos que dirigen su expresión a lo largo del desarrollo. Sus cepas mutantes presentan fenotipos durante el crecimiento y el desarrollo, de acuerdo con su complejo patrón de expresión.

3.- SrfB está implicado en varios procesos dependientes del citoesqueleto de actina tales como citocinesis y macropinocitosis durante la fase de crecimiento celular, o migración y adhesión celular durante la agregación. Además, srfB está implicado en la transición crecimiento/desarrollo y en la diferenciación de la región pstAB de las estructuras en desarrollo.

4.- El gen *acaA*, que codifica la Adenilato Ciclasa A, se transcribe desde tres promotores alternativos que son activos en varias regiones de la estructura a lo largo del desarrollo. Este patrón de expresión podría indicar que AcaA está implicada en varias etapas del desarrollo, además de su conocido papel en agregación celular.

5.- srfB regula la expresión de *acaA* en células pre-tallo situadas en la region pstAB y en células pre-espora. En este último caso la regulación podría estar mediada por señales extracelulares provenientes de la region pre-tallo.

6.- El gen *mef2A* es necesario para el correcto crecimiento de las amebas sobre bacterias. También está implicado en procesos de diferenciación celular, al igual que *srfA* y *srfB*. En concreto, *mef2A* está implicado en la diferenciación de células pre-espora y de una población de células pre-tallo.



Bibliografía

BIBLIOGRAFÍA

1. **Agarwal, A., Parrish, SN., Blumberg DD.** 1999. Ribosomal protein gene expression is cell type specific during development in Dictyostelium discoideum. Differentiation **65:**73-88.
2. **Althoefer, H., Schleiffer, A., Wassmann, K., Nordheim, A., Ammerer, G.** 1995. Mcm1 is required to coordinate G2-specific transcription in Saccharomyces cerevisiae. Mol Cell Biol. **15:**5917-28.
3. **Alvarez_Curto, E., Rozen, D., Ritchie, A., Fouquet, C., Baldauf, S. L., and Schaap, P.** 2005. Evolutionary origin of cAMP-based chemoattraction in the social amoebae. Proc Natl Acad Sci U S A **102:**6385-6390.
4. **Arnold, M., Kim, Y., Czubryt, MP., Phan, D., McAnally, J., Qi, X., Shelton, JM., Richardson, JA., Bassel-Duby, R., Olson, EN.** 2007. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell **12:**377-89.
5. **Arsenian, S., Weinhold, B,, Oelgeschläger, M., Rüther, U., Nordheim, A.** 1998. Serum response factor is essential for mesoderm formation during mouse embryogenesis. Embo J **17:**6289-99.
6. **Bai S, N., MW., Wang, B., Hsu, SH., Datta, J., Kutay, H., Yadav, A., Nuovo, G., Kumar, P., Ghoshal, K.** 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem **284:**32015-27.
7. **Baldauf, S. L.** 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science **290:**972-977.
8. **Benabentos, R., Hirose, S., Sucgang, R., Curk, T., Katoh, M., Ostrowski, EA., Strassmann, JE., Queller, DC., Zupan, B., Shaulsky, G., Kuspa, A.** 2009. Polymorphic members of the lag gene family mediate kin discrimination in Dictyostelium. Curr Biol **19:**567-72.
9. **Berks, M., Kay, R R.** 1990. Combinatorial control of cell differentiation by cAMP and DIF-1 during development of Dictyostelium discoideum. Development **110:**977-984.
10. **Black, B., Olson, E. N.** 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol **14:**167-96.
11. **Bominaar, A. A., and van Haastert, P. J. M.** 1994. Phospholipase C in Dictyostelium discoideum - identification of stimulatory and inhibitory surface receptors and G-proteins. Biochem. J. **297:**189-193.
12. **Bonner, J. T., Barkley, D., SHall, E. M., Konijn, T. M., Mason, J.W., O'Keefe, III. G., Wolfe, P. B.** 1969. Acrasin, acrasinase and the sensitivity to acrasin in Dictyostelium discoideum. Dev. Biol. **20:**72-87.
13. **Bonner, J. T., Barkley, D., SHall, E. M., Konijn, T. M., Mason, J.W., O'Keefe, III. G., Wolfe, P. B.** 1950. The orientation to light and the extremely sensitive orientation to temperature gradients in the slime mold Dictyostelium discoideum. J. Cell. Compar. Physiol. **36:**149-158.
14. **Bosgraaf, L., Russcher, H., Smith, J L., Wessels, D., Soll, D R., van Haastert, P J M.** 2002. A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in Dictyostelium. EMBO J. **21:**4560-4570.

15. **Brefeld, O.** 1869. Dictyostelium mucoroides. Ein neuer Organismus aus der Verwandtschaft der Myxomyceten. Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft Frankfurt **7:**85-107.
16. **Brown, J. M.** 2000. Functional and regulatory analyses of the Dictyostelium discoideum G-box binding factor. PhD. University of California, San Diego (UCSD), La Jolla, CA.
17. **Bush, J., Richardson, J., and Cardelli, J.** 1994. Molecular cloning and characterization of the full-length cDNA encoding the developmentally regulated lysosomal enzyme beta-glucosidase in Dictyostelium discoideum. J. Biol. Chem. **269:**1468-1476.
18. **Camoretti-Mercado B, D. N., Solway J.** 2003. Serum response factor function and dysfunction in smooth muscle. Respir Physiol Neurobiol. **137:**223-35.
19. **Camoretti-Mercado, B. L., H. W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E., Patel, N. M., Hershenson, M. B., Fuchs, E., Sinha, S., Miano, J. M., Parmacek, M. S., Burkhardt, J. K., Solway, J.** 2000. Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. J Biol Chem **275:**30387-93.
20. **Cardelli, J.** 2001. Phagocytosis and macropinocytosis in Dictyostelium: phosphoinositide-based processes, biochemically distinct. Traffic **2:**311-320.
21. **Chai, J., and Tarnawski, A. S.** 2002. Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. J Physiol Pharmacol **53:**147-57.
22. **Chang, S., McKinsey, TA., Zhang, CL., Richardson, JA., Hill, JA., Olson, EN.** 2004. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol **24:**8467-76.
23. **Chen, M. Y., Long, Y., Devreotes, P N.** 1997. A novel cytosolic regulator, pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in Dictyostelium. Genes Devel. **11:**3218-3231.
24. **Choi, H., Kim ,KR., Lee, JH., Park, HS., Jang, KY., Chung, MJ., Hwang, SE., Yu, HC., Moon, WS.** 2009. Serum response factor enhances liver metastasis of colorectal carcinoma via alteration of the E-cadherin/beta-catenin complex. Oncol Rep **21:**57-63.
25. **Chung, C., Firtel, RA.** 2002. Signaling pathways at the leading edge of chemotaxing cells. Journal of Muscle Research and Cell Motility **23:**773–779.
26. **Clarke, M., and Gomer, R. H.** 1995. PSF and CMF, autocrine factors that regulate gene expression during growth and early development of Dictyostelium. Experientia **51:**1124-1134.
27. **Clarke M, K. S., Riley K.** 1987. Density-dependent induction of discoidin-I synthesis in exponentially growing cells of Dictyostelium discoideum. Differentiation **34:**79-87.
28. **Clarke, M., Yang, J., and Kayman, S.** 1988. Analysis of the prestarvation response in growing cells of Dictyostelium discoideum. Dev. Genet. **9:**315-326.
29. **Datta R, R. E., Sukhatme, V., Qureshi, S., Hallahan, D., Weichselbaum, RR., Kufe, DW.** 1992. Ionizing radiation activates transcription of the EGR1 gene via CArG elements. Proc Natl Acad Sci U S A **89:**10149-53.
30. **de la Roche, M. A., Cote, G P.** 2001. Regulation of Dictyostelium myosin I and II. Biochim. Biophys. Acta **1525:**245-261.

31. **Ding W, G. S., Scott RE.** 2001. Senescence represses the nuclear localization of the serum response factor and differentiation regulates its nuclear localization with lineage specificity. J Cell Sci **114:**1011-8.
32. **Dodou, E., Treisman, R.** 1997. The Saccharomyces cerevisiae MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. Mol Cell Biol **17:**1848-59.
33. **Dubin, M., Kasten, S., Nellen, W.** 2011. Characterization of the Dictyostelium homolog of chromatin binding protein DET1 suggests a conserved pathway regulating cell type specification and developmental plasticity. Eukaryot Cell **10:**352-62.
34. **Edmondson, D., Lyons, GE., Martin, JF., Olson, EN.** 1994. Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. Development **120:**1251-63.
35. **Eichinger, L., Pachebat, J A., Glockner, G., Rajandream, MA., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q.** 2005. The genome of the social amoeba Dictyostelium discoideum. Nature **435:**43-57.
36. **Escalante, R., and Sastre, L.** 1998. A serum response factor homolog is required for spore differentiation in Dictyostelium. Development **125:**3801-3808.
37. **Escalante, R., and Vicente, J. J.** 2000. Dictyostelium discoideum: a model system for differentiation and patterning. Int. J. Dev. Biol. **44:**819- 835.
38. **Escalante, R., Vicente, J. J., Moreno, N., and Sastre, L.** 2001. The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of Dictyostelium development. Dev Biol **235:**314-29.
39. **Escalante, R., Yamada, Y., Cotter, D., Sastre, L., and Sameshima, M.** 2004. The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during *Dictyostelium* sporulation. Mechanisms of Development **121:**51-56.
40. **Faure, M., Franke, J., Hall, A. L., Podgorski, G. J., and Kessin, R. H.** 1990. The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum contains 3 promoters specific for growth, aggregation, and late development. Mol. Cell. Biol. **10:**1921-1930.
41. **Firtel, R. A.** 1995. Integration of signaling information in controlling cell- fate decisions in Dictyostelium. Genes Devel. **9:**1427-1444.
42. **Franca-Koh, J., Sedore Willard, S., Devreotes P.** 2010. G-Protein Signaling in Chemotaxis, p. 1705-1712. *In* R. A. B. a. E. A. Dennis (ed.), Handbook of cell signaling.
43. **Francis, D. W.** 1964. Some studies on phototaxis of Dictyostelium. J. Cell. Compar. Physiol. **64:**131-138.
44. **Fukuzawa, M., Hopper, N., Williams, J.** 1997. cudA: A Dictyostelium gene with pleiotropic effects on cellular differentiation and slug behaviour. Development **124:**2719-2728.
45. **Galardi-Castilla M, G. A., Suarez T, Sastre L.** 2010. The Dictyostelium discoideum acaA gene is transcribed from alternative promoters during aggregation and multicellular development. PLoS One **5:**e13286.
46. **Galardi-Castilla M, P. B., Bloomfield G, Skelton J, Ivens A, Kay RR, Bozzaro S, Sastre L.** 2008. SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in Dictyostelium. Dev Biol. 2008 **316:**260-74.

47. **Gauthier-Rouviere, C., Vandromme, M., Lautredou, N., Cai, Q. Q., Girard, F., Fernandez, A., Lamb, N.** 1995. The serum response factor nuclear localization signal: general implications for cyclic AMP-dependent protein kinase activity in control of nuclear translocation. Mol Cell Biol **15:**433-44.
48. **Gerisch, G.** 1987. Cyclic AMP and other signals controlling cell development and differentiation in Dictyostelium. Annu. Rev. Biochem. **56:**853-879.
49. **Gollop, R., and Kimmel, A. R.** 1997. Control of cell-type specific gene expression in Dictyostelium by the general transcription factor GBF. Development **124:**3395-3405.
50. **Gomer, R. H., Yuen, I S., Firtel, R A.** 1991. A secreted 80x10(3)Mr protein mediates sensing of cell density and the onset of development in Dictyostelium. Development **112:**269-278.
51. **Greenberg, M., Ziff, EB.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature **311:**433-8.
52. **Greenberg ME, S. Z., Ziff EB.** 1987. Mutation of the c-fos gene dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol Cell Biol **7:**1217-25.
53. **Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M., and Krasnow, M. A.** 1996. The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. Development **122:**1353-62.
54. **Hall, A. L., Franke, J., Faure, M., and Kessin, R. H.** 1993. The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde. Dev. Biol. **157:**73-84.
55. **Han, J., Jiang, Y., Li, Z., Kravchenko, VV., Ulevitch, RJ.** 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. Nature **386:**296-9.
56. **Hjorth, A. L., Pears, C., Williams, J G., Firtel, R A.** 1990. A developmentally regulated trans-acting factor recognizes dissimilar G/C-rich elements controlling a class of cAMP-inducible Dictyostelium genes. Genes Devel. **4:**419-432.
57. **Insall, R., Kuspa, A., Lilly, P J., Shaulsky, G., Levin, L R., Loomis, W F., Devreotes, P.** 1994. CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in Dictyostelium. J. Cell Biol. **126:**1537-1545.
58. **Iranfar, N., Fuller, D., and Loomis, W.F.** 2003. Gene regulation during early development of *Dictyostelium* using genome-wide expression analyses. Eukaryotic Cell **2:**664-670.
59. **Janknecht R, H. R., Houthaeve T, Nordheim A, Stunnenberg HG.** 1992. Identification of multiple SRF N-terminal phosphorylation sites affecting DNA binding properties. Embo J **11:**1045-54.
60. **Kaplan-Albuquerque, N., Van Putten, V., Weiser-Evans, MC., Nemenoff, RA.** 2005. Depletion of serum response factor by RNA interference mimics the mitogenic effects of platelet derived growth factor-BB in vascular smooth muscle cells. Circ Res **97:**427-33.
61. **Kato, Y., , Kravchenko, VV., Tapping, RI., Han, J., Ulevitch, RJ., Lee, JD.** 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. EMBO J **16:**7054-66.
62. **Kay, R. R., Flatman, P., Thompson, C R L.** 1999. DIF signalling and cell fate. Sem. Cell Dev. Biol. **10:**577-585.

63. **Kay, R. R., Thompson, C R L.** 2001. Cross-induction of cell types in Dictyostelium: evidence that DIF-1 is made by prespore cells. Development **128:**4959-4966.
64. **Kemp, P. R., Metcalfe, J. C.** 2000. Four isoforms of serum response factor that increase or inhibit smooth-muscle-specific promoter activity. Biochem J **345 Pt 3:**445-51.
65. **Ken, R., and Singleton, C. K.** 1994. Redundant regulatory elements account for the developmental control of a ribosomal protein gene of Dictyostelium discoideum. Differentiation **55:**97-103.
66. **Kollmar, M.** 2006. Thirteen is enough: the myosins of Dictyostelium discoideum and their light chains. BMC Genomics **7:**183-198.
67. **Konijn, T. M., Chang, Y Y., Bonner, J T.** 1969. Synthesis of cyclic AMP in Dictyostelium discoideum and Polysphondylium pallidum. Nature **224:**1211-1212.
68. **Kriebel, P., Parent, CA.** 2004. Adenylyl cyclase expression and regulation during the differentiation of Dictyostelium discoideum. **56:**541-6.
69. **Landerholm, T. E., Dong, X. R., Lu, J., Belaguli, N. S., Schwartz, R. J., Majesky, M. W.** 1999. A role for serum response factor in coronary smooth muscle differentiation from proepicardial cells. Development **126:**2053-62.
70. **Li, S., Czubryt, M P., McAnally, J., Bassel-Duby, R., Richardson, J A., Wiebel, F. F., Nordheim, A., Olson, E N.** 2005. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. Proc Natl Acad Sci U S A **102:**1082-1087.
71. **Lilly B, G. S., Firulli AB, Schulz RA, Olson EN.** 1994. D-MEF2: aMADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during Drosophila embryogenesis. Proc Natl Acad Sci USA **91:**5662–5666.
72. **Liu, S., Ma, JT., Yueh, AY., Lees-Miller, SP., Anderson, CW., Ng, SY.** 1993. The carboxyl-terminal transactivation domain of human serum response factor contains DNA-activated protein kinase phosphorylation sites. J Biol Chem **268:**21147-54.
73. **Loomis, W., Shaulsky, G.** 2011. Developmental changes in transcriptional profiles. Dev Growth Differ **53:**567-75.
74. **Loomis, W. F., Smith, D W.** 1995. Consensus phylogeny of Dictyostelium. Experientia **51:**1110-1115.
75. **Louis, J. M., Saxe III, C L., Kimmel, A R.** 1993. Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during Dictyostelium development. Proc. Natl. Acad. Sci. USA **90:**5969-5973.
76. **Lu, J., McKinsey, TA., Zhang, CL., Olson, EN.** 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Mol Cell **6:**233-4.
77. **Lu, X. G., Azhar, G., Liu, L., Tsou, H., Wei, J. Y.** 1998. SRF binding to SRE in the rat heart: influence of age. J Gerontol A Biol Sci Med Sci **53:**B3-10.
78. **MacWilliams, H., Doquang, K., Pedrola, R., Dollman, G., Grassi, D., Peis, T., Tsang, A., Ceccarelli, A.** 2006. A retinoblastoma ortholog controls stalk/spore preference in Dictyostelium. Development **133:**1287-97.
79. **Maeda, Y.** 2005. Regulation of growth and differentiation in Dictyostelium. International Review of Cytology **244:**287-332.
80. **Manahan, C., Iglesias, PA., Long, Y., Devreotes, PN.** 2004. Chemoattractant signaling in dictyostelium discoideum. Annu Rev Cell Dev Biol **20:**223-53.

81. **Manak JR, d. B. N., Kris RM, Prywes R.** 1990. Casein kinase II enhances the DNA binding activity of serum response factor. Genes Dev **4:**955-67.
82. **Manak JR, P. R.** 1991. Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by casein kinase II. Mol Cell Biol. **11:**3652-9.
83. **Martin, J., Miano, JM., Hustad, CM., Copeland, NG., Jenkins, NA., Olson, EN.** 1994. A Mef2 gene that generates a muscle-specific isoform via alternative mRNA splicing. **14:**1647-56.
84. **McInerny, C., Partridge, JF., Mikesell, GE., Creemer, DP., Breeden, LL.** 1997. A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription. Genes Dev **11:**1277-88.
85. **McKinsey, T., Zhang, CL., Olson, EN.** 2001. Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev **11:**497-504.
86. **McKinsey, T., Zhang, CL., Olson, EN.** 2002. MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci **27:**40-7.
87. **Mead, J., Bruning, AR., Gill, MK., Steiner, AM., Acton, TB., Vershon, AK.** 2002. Interactions of the Mcm1 MADS box protein with cofactors that regulate mating in yeast. Mol Cell Biol **22:**4607-21.
88. **Medjkane S, P.-S. C., Gaggioli C, Sahai E, Treisman R.** 2009. Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. Nat Cell Biol **11:**257-68.
89. **Mehdy, M. C.** 1985. The regulation of cell-type-specific gene expression in Dictyostelium (cyclic-AMP). PhD. University of California, San Diego (UCSD), La Jolla, CA.
90. **Meili, R., Ellsworth, C., Firtel, R A.** 2000. A novel Akt/PKB-related kinase is essential for morphogenesis in Dictyostelium. Curr. Biol. **10:**708-717.
91. **Messenguy, F., Dubois, E.** 2000. Regulation of Arginine Metabolism in Saccharomyces cerevisiae: a Network of Specific and Pleiotropic Proteins in Response to Multiple Environmental Signals. Food technol. biotechnol. **38:**277-285.
92. **Messenguy, F., Dubois, E.** 2003. Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene **316:**1-21.
93. **Meyyappan, M., Wheaton, K., Riabowol, K. T.** 1999. Decreased expression and activity of the immediate-early growth response (Egr-1) gene product during cellular senescence. J Cell Physiol **179:**29-39.
94. **Miano, J. M.** 2003. Serum response factor: toggling between disparate programs of gene expression. Journal of Molecular cell Cardiology **137:**223-235.
95. **Miano, J. M., Long X., Fujiwara, K.** 2007. Serum response factor: master regulator of the actin cytoskeleton and contractil apparatus. Am J Physiol Cell Physiol **292:**C70-C81.
96. **Miano, J. M., Ramanan, N., Georger, M A., de Mesy-Bentley, K L., Emerson, R L., Balza, R O Jr.,Xiao, Q., Weiler, H., Ginty, D. D., Misra, R. P.** 2004. Restricted inactivation of serum response factor to the cardiovascular system. Proc Natl Acad Sci U S A **101:**17132-17137.

97. **Misra, R. P., Rivera, V. M., Wang, J. M., Fan, P. D., Greenberg, M. E.** 1991. The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. Mol Cell Biol **11:**4545-54.
98. **Mo, Y., Ho, W., Johnston, K., Marmorstein, R.** 2001. Crystal structure of a ternary SAP-1/SRF/c-fos SRE DNA complex. J Mol Biol **314:**495-506.
99. **Montagne, J., Groppe, J., Guillemin, K., Krasnow, M. A., Gehring, W. J., Affolter, M.** 1996. The Drosophila Serum Response Factor gene is required for the formation of intervein tissue of the wing and is allelic to blistered. Development **122:**2589-97.
100. **Nguyen, H., Hadwiger, JA.** 2009. The Galpha4 G protein subunit interacts with the MAP kinase ERK2 using a D-motif that regulates developmental morphogenesis in Dictyostelium. Dev Biol. **335:**385-95.
101. **Niu, Z., Yu, W., Zhang, SX., Barron, M., Belaguli, NS., Schneider, MD., Parmacek, M., Nordheim, A., Schwartz, RJ.** 2005. Conditional mutagenesis of the murine serum response factor gene blocks cardiogenesis and the transcription of downstream gene targets. J Biol Chem **280:**32531-8.
102. **Norman, C., Runswick, M., Pollock, R., Treisman, R.,.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55:**989-1003.
103. **Ogawa S, Y. R., Angata K, Iwamoto M, Pi M, Kuroe K, Matsuo K, Morio T, Urushihara H, Yanagisawa K, Tanaka Y.** 2000. The mitochondrial DNA of Dictyostelium discoideum: complete sequence, gene content and genome organization. Mol Gen Genet **263:**514-9.
104. **Pan, P., Hall, E M., Bonner, J T.** 1972. Folic acid as second chemotactic substance in the cellular slime moulds. Nature New Biol. **237:**181-182.
105. **Park, M., Kim, KR., Park, HS., Park, BH., Choi, HN., Jang, KY., Chung, MJ., Kang, MJ., Lee, DG., Moon, WS.** 2007. Expression of the serum response factor in hepatocellular carcinoma: implications for epithelial-mesenchymal transition. Int J Oncol **31:**1309-15.
106. **Parlakian, A., Tuil, D., Hamard, G., Tavernier, G., Hentzen, D., Concordet, JP., Paulin, D., Li, Z., Daegelen D.** 2004. Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. Mol Cell Biol **24:**5281-9.
107. **Pipes, G. C. T., Creemers, E.E., Olson, E N.** 2006. The myocardin family or transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. Genes & Develop. **20:**1545-1556.
108. **Poff, K. L., Hader, D P.** 1984. An action spectrum for phototaxis by pseudoplasmodia of Dictyostelium discoideum. Photochem. Photobiol. **39:**433-436.
109. **Poff, K. L., Skokut, M.** 1977. Thermotaxis by pseudoplasmodia of Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA **74:**2007-2010.
110. **Pollock, R., Treisman, R.** 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev **5:**2327-41.
111. **Potthoff, M., Olson, E N.** 2007. MEF2: a central regulator of diverse developmental programs. Development **134:**4131-4140.
112. **Prywes R, R. R.** 1986. Inducible binding of a factor to the c-fos enhancer. Cell **47:**777-84.
113. **Raper, K. B.** 1935. Dictyostelium discoideum, a new species of slime mold from decaying forest leaves. J. Agr. Res. **50:**135-147.

114. **Romeralo, M., Escalante, R., Baldauf, SL.** 2011. Evolution and Diversity of Dictyostelid Social Amoebae. Protist **163:**327-43.
115. **Sawarkar, R., Visweswariah, SS., Nellen, W., Nanjundiah, V.** 2009. Histone deacetylases regulate multicellular development in the social amoeba Dictyostelium discoideum. J Mol Biol **391:**833-48.
116. **Schnitzler, G. R., Fischer, W H., Firtel, R A.** 1994. Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in Dictyostelium. Genes Devel. **8:**502-514.
117. **Schratt G, P. U., Berger J, Schwarz H, Heidenreich O, Nordheim A.** 2002. Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. J Cell Bio **156:**737-50.
118. **Schulkes, C., Schaap, P.** 1995. cAMP-dependent protein kinase activity is essential for preaggregative gene expression in Dictyostelium. FEBS Lett. **368:**381-384.
119. **Shore, P., Sharrocks, A. D.** 1995. The MADS-box family of transcription factors. Eur J Biochem **229:**1-13.
120. **Sillo, A., Bloomfield, G., Balest, A., Balbo, A., Pergolizzi, B., Peracino, B., Skelton, J., Ivens, A., Bozzaro, S.** 2008. Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in Dictyostelium. BMC Genomics**:**1-22.
121. **Siol, O., Dingermann, T., Winckler, T.** 2006. The C-module DNA-binding factor mediates expression of the dictyostelium aggregation-specific adenylyl cyclase ACA. Eukaryot Cell **5:**658-64.
122. **Siu, C., Harris, TJ., Wang, J., Wong, E.** 2004. Regulation of cell-cell adhesion during Dictyostelium development. Semin Cell Dev Biol **15:**633-41.
123. **Soulez, M., Tuil, D., Kahn, A., Gilgenkrantz, H.** 1996. The serum response factor (SRF) is needed for muscle-specific activation of CArG boxes. Biochem Biophys Res Commun **219:**418-22.
124. **Souza, G. M., da Silva, A M., Kuspa, A.** 1999. Starvation promotes Dictyostelium development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. Development **126:**3263-3274.
125. **Srinivasan, S., Alexander, H., Alexander, S.** 2000. Crossing the finish line of development: regulated secretion of Dictyostelium proteins. Trends Cell Biol. **10:**215-219.
126. **Sun, T. J., Devreotes, P N.** 1991. Gene targeting of the aggregation stage cAMP receptor cAR1 in Dictyostelium. Genes Devel. **5:**572-582.
127. **Swanson, A. R., Vadell, E M., Cavender, J C.** 1999. Global distribution of forest soil dictyostelids. J. Biogeography **26:**133-148.
128. **Treisman, R.** 1987. Identification and purification of a polypeptide that binds to the c-fos serum response element. Embo J **6:**2711-7.
129. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Semin Cancer Biol **1:**47-58.
130. **Verkerke-van Wijk, I., Fukuzawa, M., Devreotes, P N., Schaap, P.** 2001. Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression. Dev. Biol. **234:**151-160.
131. **Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N.** 2001. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell **105:**851-62.

132. **Wang, D. Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A., Olson, E. N.** 2002. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc Natl Acad Sci U S A **99:**14855-60.

133. **Wang, Z., Wang, D-Z., Nockemeyer, D., McAnally, J., Nordheim, A., Olson, E N.** 2004. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature **428:**185-189.

134. **Wei, L., Zhou, W., Croissant, J. D., Johansen, F. E., Prywes, R., Balasubramanyam, A., and Schwartz, R. J.** 1998. RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. J Biol Chem **273:**30287-94.

135. **Williams, J. G.** 2006. Transcriptional regulation of Dictyostelium pattern formation. EMBO Rep. **7:**694-698.

136. **Winckler, T., Iranfar, N., Beck, P., Jennes, I., Siol, O., Baik, U., Loomis, WF., Dingermann, T.** 2004. CbfA, the C-module DNA-binding factor, plays an essential role in the initiation of Dictyostelium discoideum development. Eukaryot Cell **3:**1349-58.

137. **Wu, L. J., Valkema, R., van Haastert, P J M., Devreotes, P N.** 1995. The G protein beta subunit is essential for multiple responses to chemoattractants in Dictyostelium. J. Cell Biol. **129:**1667-1675.

138. **Yang, C., Ornatsky, OI., McDermott, JC., Cruz, TF., Prody, CA.** 1998. Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen-activated protein kinase, ERK5/BMK1. Nucleic Acids Res **26:**4771-7.

139. **Yuen, I. S., Jain, R., Bishop, J D., Lindsey, D F., Deery, W J., van Haastert, P J M., Gomer, R H.** 1995. A density-sensing factor regulates signal transduction in Dictyostelium. J. Cell Biol. **129:**1251-1262.

140. **Zhang, X., Azhar, G., Chai, J., Sheridan, P., Nagano, K., Brown, T., Yang, J., Khrapko, K., Borras, A. M., Lawitts, J., Misra, R. P., Wei, J. Y.** 2001. Cardiomyopathy in transgenic mice with cardiac-specific overexpression of serum response factor. Am J Physiol Heart Circ Physiol **280:**H1782-92.

141. **Zhou, J., Hu, G., Herring, BP.** 2005. Smooth muscle-specific genes are differentially sensitive to inhibition by Elk-1. Mol Cell Biol **25:**9874-85.

Anexos

ANEXOS

Artículo 1: *Structural and functional studies of a family of Dictyostelium discoideum developmentally regulated, prestalk genes coding for small proteins.*

Dictyostelium discoideum lleva a cabo un programa de desarrollo multicelular bajo condiciones de ayuno. Este proceso morfogenético requiere de la regulación diferencial de un gran número de genes y está coordinado por señales extracelulares. El factor de transcripción de la familia MADS, srfA, está implicado en varios estadios del desarrollo incluyendo migración del *"slug"* y diferenciación terminal de las esporas.

Mediante hibridación substractiva se aisló el gen, *sigN* (*"Srf-A-induced gene N"*) o gen inducido por srfA N, cuya expresión en el estadio de *"slug"* es dependiente del factor de transcripción SrfA. Las búsquedas por homología detectaron la existencia de una gran familia de genes relacionados con *sigN* en el genoma de *D. discoideum*. Los 13 genes más parecidos están agrupados en dos regiones del cromosoma 2 y fueron denominados genes *sigN* de Grupo 1 y de Grupo 2. Las proteínas codificadas tienen unos 87-89 aminoácidos. Todos los genes presentan una estructura similar, compuesta por un primer exón que contiene una pauta abierta de lectura de 13 nucleótidos y un segundo exón que contiene el resto de la región codificante. La expresión de estos genes es inducida a las 10 horas de desarrollo. El análisis de sus regiones promotoras indica que estos genes se expresan en la región pre-tallo de las estructuras en desarrollo. La adición de anticuerpos contra las proteínas SigN del Grupo 2 provoca que las estructuras en estadio "*mound"* se descompongan.

En conclusión se ha encontrado en *D. discoideum* una gran familia de genes que codifican proteínas de pequeño tamaño. Dos de los grupos con proteínas muy similares se expresan específicamente en células pre-tallo durante el desarrollo. Los estudios funcionales con anticuerpos contra las proteínas SigN del Grupo 2, vislumbran un posible papel de estos genes durante el desarrollo pluricelular.

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Structural and functional studies of a family of *Dictyostelium discoideum* developmentally regulated, prestalk genes coding for small proteins

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Abstract

Background: The social amoeba *Dictyostelium discoideum* executes a multicellular development program upon starvation. This morphogenetic process requires the differential regulation of a large number of genes and is coordinated by extracellular signals. The MADS-box transcription factor SrfA is required for several stages of development, including slug migration and spore terminal differentiation.

Results: Subtractive hybridization allowed the isolation of a gene, *sigN* (SrfA-induced gene N), that was dependent on the transcription factor SrfA for expression at the slug stage of development. Homology searches detected the existence of a large family of *sigN*-related genes in the *Dictyostelium discoideum* genome. The 13 most similar genes are grouped in two regions of chromosome 2 and have been named Group1 and Group2 *sigN* genes. The putative encoded proteins are 87–89 amino acids long. All these genes have a similar structure, composed of a first exon containing a 13 nucleotides long open reading frame and a second exon comprising the remaining of the putative coding region. The expression of these genes is induced at10 hours of development. Analyses of their promoter regions indicate that these genes are expressed in the prestalk region of developing structures. The addition of antibodies raised against SigN Group 2 proteins induced disintegration of multi-cellular structures at the mound stage of development.

Conclusion: A large family of genes coding for small proteins has been identified in *D. discoideum*. Two groups of very similar genes from this family have been shown to be specifically expressed in prestalk cells during development. Functional studies using antibodies raised against Group 2 SigN proteins indicate that these genes could play a role during multicellular development.

Background

The social amoeba *Dictyostelium discoideum* is one of the simplest model systems utilized for the study of multi-cellular development. This organism lives as individual amoeba on forest soils, feeding on other microorganisms. However, when their food source is exhausted, they aggregate in groups of up to 100,000 cells and initiate a multicellular developmental program to form a fruiting body that stands on the substrate (reviewed in [1]). At the top of the fruiting body, inside the sorus, a large proportion of

the original amoeba differentiate into resistant forms, called spores, that stay alive for long periods of time. Spores disseminate in the media and germinate to give rise to new amoeba when they reach favourable environmental conditions.

Aggregation of the amoebae is directed by chemotaxis to cAMP, secreted from discrete aggregation centres (reviewed in [2]). Cells that converge towards aggregation centres adhere among them forming small mounds covered by an extracellular matrix [3]. Cell-cell adhesion is mediated by several membrane proteins, whose expression is induced during development. The first known cell-cell adhesion system to be induced, soon after starvation, is Ca-dependent and is composed of the homophilic protein DdCAD-1 (gp24), encoded by the gene *cadA*[4]. A second homophilic, EDTA-resistant, adhesion system is induced at the onset of aggregation and is composed by the gp80 protein, encoded by the *csaA* gene [5,6]. A third adhesion system is induced later during aggregation being mediated by the gp150 proteins, encoded by the gene *lag*C [7]. Mutations in some of the genes coding for these adhesion systems or experimental conditions that interfere with their function, compromise the formation or stability of the multicellular structure [8-10].

Cells in the aggregates follow two alternative differentiation programs to become prestalk or prespore cells. At the same time, these cells continue to move towards cAMP secreted from the centre of the mound. Differences in chemotaxis to cAMP and in cell adhesion mediate the segregation of cell types so that prestalk cells migrate centrally and upwards to form a small protrusion, or tip, at the upper part of the structure [11]. This organization is maintained during most of development, including a migratory structure, the slug, that is formed under particular environmental conditions. Coordinated cell movement and differentiation continues during the rest of the morphogenetic process when prestalk cells move downwards to the substrate and differentiate to form the stalk. In the meanwhile, prespore cells migrate upwards to form the sorus where they differentiate to spores.

Morphogenesis is coordinated through the secretion of different factors that regulate cell adhesion, migration and differentiation, cAMP being the most important among them. The chlorinated alkyl phenone DIF-1 is also an important regulator of stalk cell differentiation [12]. Other cell-signalling factors are peptides or small proteins that are secreted by some cells to regulate the activity of neighbouring cells. For example, countin (258 amino acids long) and D11 (284 amino acids, encoded by the gene *ampA*) are secreted proteins that inhibit cell adhesion and contribute to regulate the size of the structure [13,14]. Also two secreted peptides, SDF-1 and SDF-2, originated by proteolysis of the AcbA protein, induce spore differentiation at culmination [15].

Dictyostelium multi-cellular development is also dependent on the co-ordinated regulation of gene expression. Multiple genes are either inhibited or induced at different stages of development [16]. This regulation is obtained by the activity of several transcription factors whose expression, or activity, is regulated during development [17]. One of the best-known transcription factors that regulates development is GBF (G-box binding factor), that is required for induction of many prestalk and prespore genes in the mound. For example, expression of the gene lagC, coding for the adhesion protein gp150, is dependent on GBF [18].

The MADS-box transcription factor SrfA is also involved in the regulation of multi-cellular development. Strains where the *srfA* gene has been knocked out showed defects in slug migration, morphogenesis and spore differentiation [19,20]. The identification of genes regulated by SrfA and involved in these processes can be an interesting approach to the study of development. Twenty four SrfA-dependent genes specifically expressed during spore differentiation have been described previously [21,22]. This article describes the isolation of SrfA-dependent genes expressed at the slug stage of development and focused on one of them, *sigN* (SrfA-induced gene N). This gene belongs to an extensive family of genes coding for small proteins, containing less than 100 amino acids, which are expressed in the prestalk region and seem to be involved in maintaining the integrity of the cell aggregates.

Methods

Cell culture, transformation and development

Dictyostelium cells were grown axenically in HL5 medium [23]. Transformations were carried out as described by Kuspa and Loomis [24]. Transformed cells were selected by treatment with blasticidine [25] or neomycin (GP418). Transformants were grown on SM plates in association with *Klebsiella aerogenes* for clonal isolation [23]. Development on nitrocellulose filters was performed as described by Shaulsky and Loomis [26].

Subtractive library construction

RNA was isolated from slug structures of Wild Type (AX4) and *srfA*$^-$ strains using Trizol reagent (Gibco-BRL). Four micrograms of poly(A)$^+$RNA, purified using a mRNA purification kit (GEHealthcare), were used for synthesis of a cDNA subtraction library using a PCR-Select cDNA subtraction kit (Clontech), as previously described [21]. Wild-type cDNA was used as the tester cDNA, and *srfA*$^-$ cDNA as the driver cDNA. Subtracted cDNAs were cloned in the pGEMT-Easy vector (Promega).

Analyses of the promoter regions

Putative promoter regions of the *sigN2* and *sigN9* genes were amplified by PCR and cloned in the PsA-ialphagal vector [27], in substitution of the PsA promoter (excised as a XbaI-BglII fragment). Reporter vectors were transfected in AX4 cells by electroporation and the transformed colonies selected by geneticin (GP418) resistance. β-galactosidase activity was detected on developing structures as previously described [28].

Generation of plasmid vectors

The plasmid vector used for RNA interference was based on exon 2 of *sigN3*. This exon was amplified by PCR and introduced in a pGEMT-Easy vector (Promega). This sequence was used to amplify the reverse complement. Forward and reverse fragments were cloned flanking a stuffer DNA [29] and the whole construct introduced in a *Dictyostelium* expression vector: pDXA-HC [30].

For generation of the *sigN* over-expression vector, a 300 bp fragment of the *sigN4* gene was amplified by PCR and introduced in pGEMT-Easy vector. The fragment was taken out through a HindIII/XhoI digestion, and introduced in the expression vector pDXA-HC, under the control of the actin 15 promoter.

The *sigN* Group1 KO construct was generated from two regions of 460 and 1300 bp respectively, flanking the genomic region to be deleted. These fragments were amplified by PCR and introduced in the pBlueScript vector, flanking a blasticidin-resistance cassette. Oligonucleotides 5'-CGAATTCCCTTCTAATTGGTCTAATC-3' and 5'-CGCGGCCGCGGAGTTGGTTCCATTTTAACTGG-3' were used for amplification of the 460 bp fragment and oligonucleotides 5'-CGGATCCCGCCGTGGCGGCATTAGCATTAGC-3' and 5'-GAATTCGTGAGAACAGCACTGACTTACCTCC-3' for that of the 1300 bp fragment. The *sigN* Group 2 KO vector was constructed in a similar way from two fragments of 1000 and 730 bp, flanking the genomic region to be eliminated. These fragments were generated by PCR and cloned in pBlueScript at both sites of the Blasticidin-resistance cassette. Oligonucleotides 5'-CGAATTCCTACCGATGTTTCAGCAAGAGG-3' and 5'-CGGATCCCACAGTTGGTACCATTACAAATCC-3' were used for amplification of the 1000 bp fragment and 5'-CCGGTACCCTGTCAATGGAGTTGTTGGAGG-3' and 5'-CCCTGCAGGAATCAAGAGGATCTGGTCAATTGG-3' for that of the 730 bp fragment. The double-KO of Group 1 and Group 2 genes was made by co-transfection of the plasmid vector used for generation of the Group 1 deletion and the reporter vector ialphagal, carrying the neomycin resistance gene [27], in Group 2 mutant cells. Transformed cells were selected by treatment with GP418, grown on SM plates in association with *Klebsiella aerogenes* and individual colonies analysed for deletion of Group 1 genes by PCR. Colonies that presented deletion of Group 1 and Group 2 genes were isolated.

Generation of antibodies against Group 1 and Group 2 proteins

The differences in amino acid sequence between group 1 and group 2 proteins were used to design group-specific peptides, CGSVLHGVGSILTGG (Group 1) and CGTVVGTVNGVVGGL (Group 2), used as antigens. Antibodies were generated by Genosphere Biotechnologies (Paris, France)

Immunohistochemistry and Western blotting

Cells were washed free of liquid medium spread on cover slips and fixed with 4% PBS-paraformaldehyde during 30 minutes at room temperature. Mound structures were developed on Nitrocellulose Filters and transferred to cover slips before fixation. Cells and structures were washed twice with PBS, permeabilized with chilled methanol during 2 minutes, washed again with PBS and blocked with PBS+0,2% BSA for 20 minutes. Incubation with the first antibody was made in 150 µl of blocking buffer for 1 hour. Preparations were washed 6 times (5 minutes each) with PBS+BSA before incubation with150 µl of Alexa 568-coupled secondary antibody (dilution 1/1000) for 30 minutes. Preparations were washed twice with PBS+BSA and mounted with Prolong. Images were taken with a confocal microscopy (Olympus Fluoview 1000 confocal microscope) and processed with Adobe Photoshop software.

Western blot analyses of total protein extracts from vegetative cells were made as previously described [31].

Sequence Alignments

Nucleotide and amino acid sequence alignments were performed with the ClustalW program at the San Diego Supercomputer Centre server. The alignments obtained were checked out in a local machine with the ClustalX program. Aligned sequences were used for the generation of phylogenetic trees using the CLC Free Workbench software.

Results

Identification of a large family of genes related to sigN, *a* srfA*-dependent gene*

Strains mutant for the *srfA* gene showed abnormal slug migration [20], suggesting that genes whose expression is regulated by the transcription factor SrfA could be directly involved in proper function of the slugs. The isolation of SrfA-dependent genes was approached by the synthesis of a differential cDNA library using RNA isolated from WT slugs subtracted with RNA from *srfA*- slugs. Several cDNA clones were obtained whose expression was higher in WT

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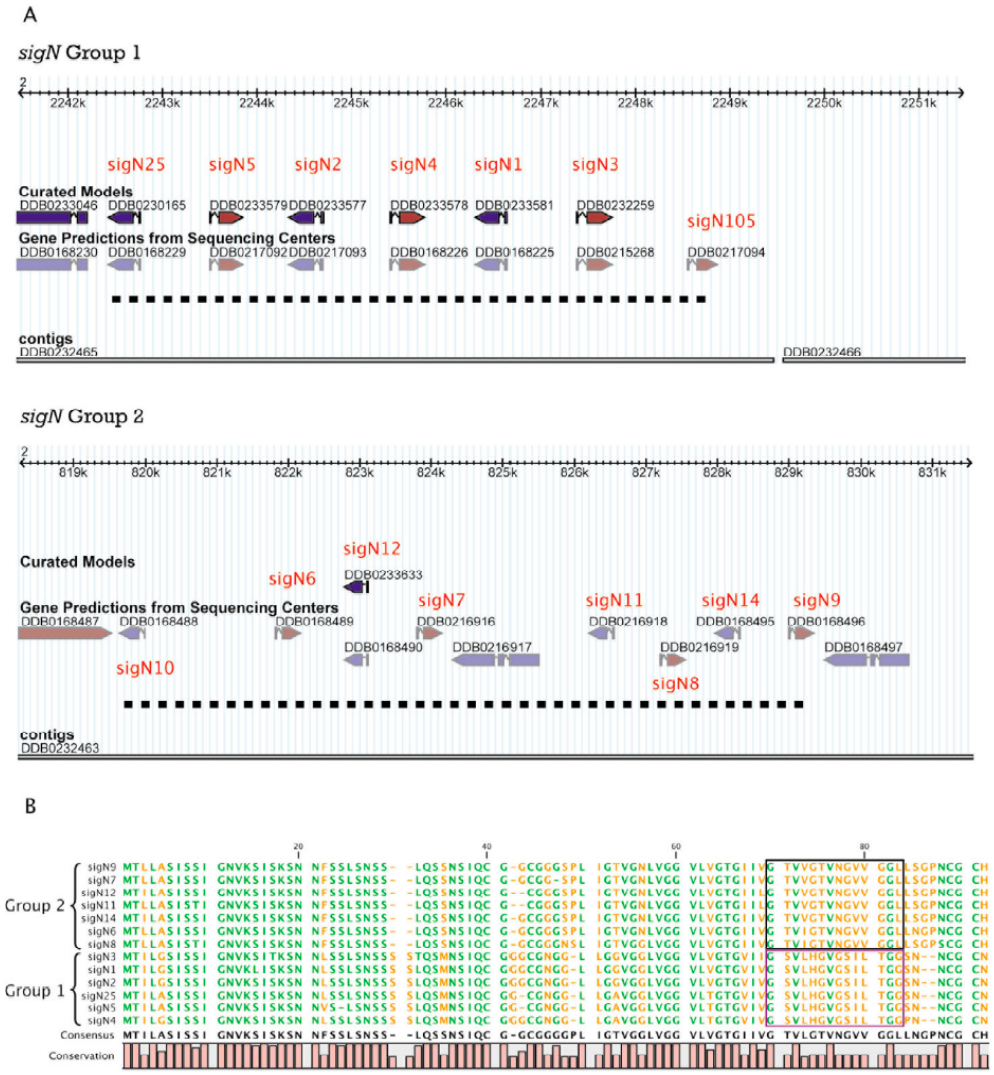

Figure 1

Chromosomal organization and similarity of Groups 1 and 2 *sigN* genes. Panel A schematically shows the location of Group 1 (upper part) and Group 2 (lower part) genes in chromosome 2. Exon coding regions are indicated as boxes and intron sequences as lines. Direction of transcription is indicated by arrowheads. Genomic fragments (contigs) where the genes are located, and the database number (curated and gene prediction) for each gene are shown in the figure. The position of exon 1 of the *sigN2* gene was modified with respect to the curated model after re-sequencing of this genomic region. Dashed lines represent the genomic regions eliminated in KO strains; from bp 819699 to 829288 for Group 2, and from bp 2242478 to 2248753 for Group 1. B. Protein sequences deduced for *sigN* genes were compared trough the use of clustal alignments. Amino acid identity is represented as bars (conservation). Amino acids conserved in all proteins are represented in green. The more divergent regions, used to generate antibodies specific for the proteins of each group are boxed.

than in *srfA*- slugs. One of them, named *sigN* (SRF-induced gene N), is described in this article.

The nucleotide sequence of the *sigN* cDNA was compared with the *D. discoideum* genome sequence. The corresponding gene is located in chromosome 2 and contains two exons separated by one small intron. The open reading frame region contained in exon 1 is 13 nucleotides long, and that of exon 2 is 254 nucleotides long. The encoded protein is, therefore, 89 amino acids long and contains a large proportion of serine cystein and glycine residues (Fig 1B). Blast searches of *Dictyostelium* SigN protein did not detect any homology with proteins in other organisms. The product of the gene *sigN*, appears in the *Dictyostelium* database as a "cysteine knot domain-containing protein". Cysteine-knot is a special folding domain found in many extracellular proteins like TGF-β2, PDGF, NGF and some neurotoxins. This kind of structure involves six conserved cysteines, generating three intramolecular disulphide bridges arranged in a knot-like topology, which gives a highly efficient motif for structure stabilization. The difference between this classic cysteine knot and the knot found in SigN1 is the number of cysteines, four instead of six, in this hypothetical protein. Several programs also predicted the existence of a putative transmembrane domain between amino acids 54 and 76 of the protein.

Homology searches in the Dictyostelium genome detected the existence of 96 genes coding for small proteins (less than 120 amino acids) with high similarity to SigN. Most of the genes (89/96) presented the same gene structure: one exon with a 13 nt long open reading frame, one intron and a second exon. The closest homologous genes are shown in Table 1 and a complete relation is shown in Additional file 1. The phylogenetic relation among these proteins is shown in Figure 2. The analyses of functional domain databases showed a hypothetical "coiled-coil" secondary structure in some of the proteins. This is a structural domain usually involved in oligomerization of proteins and is present in proteins with very different functions, which made it impossible to predict *a priori* a possible function for these proteins.

Analysis of the complete sequence of Dictyostelium genome at the DictyBase database allowed determination of the location of all these genes. The gene coding for the cDNA initially isolated and the closest homologous genes were grouped in a 7 kb long region of chromosome 2 (Fig 1A and Table 1). All these genes code for highly similar proteins (Fig 1B, Fig 2) and have been named sigN1 (the gene isolated from the subtractive library), sigN2, 3, 4, 5 and 25 (Group 1). Another group of genes with high identity to sigN1 was found in a different 10 kb long region of chromosome 2 (Fig 1A). The members of this second group showed lower identity with sigN1, between 60–

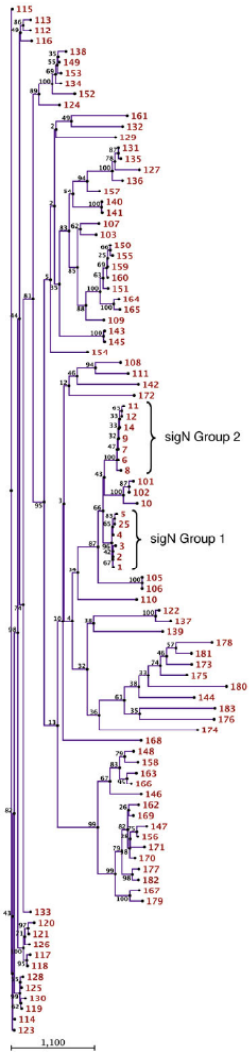

Figure 2
Phylogenetic tree of the proteins encoded by the family of *sigN* genes. The predicted amino acid sequences encoded by 96 genes that showed significant similarity to *sigN1* were compared using the ClutalW program at the online Biology WorkBench facilities from San Diego Supercomputer Center. The alignment was used to construct a phylogenetic tree using the neighbor-joining method and 100 bootstrap trials. The number of times each branch was obtained is indicated. The number assigned to each *sigN* gene is shown to the right. Group 1 and Group 2 *sigN* genes are indicated by brackets.

Table 1: *Dictyostelium discoideum* genes highly similar to *sigN1*.

Gene Name	DDB (prediction)	DDB (curated)	Chromosome	Exons number – size (bp)	Intron size (bp)	Aminoacids	Identity with sigN1 protein (%)
sigN1	DDB0168225	DDB0233581	2	1 – 13//2 – 256	66	89	100
sigN2	DDB0217093	DDB0233577	2	1 – 13//2 – 256	70	89	99
sigN3	DDB0215268	DDB0232259	2	1 – 13//2 – 256	107	89	95
sigN4	DDB0168226	DDB0233578	2	1 – 13//2 – 256	82	89	95
sigN25	DDB0168229	DDB0230165	2	1 – 13//2 – 253	71	88	95
sigN5	DDB0217092	DDB0233579	2	1 – 13//2 – 250	89	87	94
sigN7	DDB0216916		2	1 – 13//2 – 253	79	88	69
sigN12	DDB0168490	DDB0233633	2	1 – 13//2 – 253	72	88	68
sigN14	DDB0168495		2	1 – 13//2 – 256	71	89	68
sigN6	DDB0168489		2	1 – 13//2 – 256	79	89	67
sigN9	DDB0168496		2	1 – 13//2 – 256	79	89	67
sigN8	DDB0216919		2	1 – 13//2 – 256	80	89	66
sigN11	DDB0216918		2	1 – 13//2 – 253	79	88	65
sigN10	DDB0168488		2	1 – 13//2 – 256	84	89	62
sigN105	DDB0217094	DDB0238206	2	1 – 13//2 – 214	87	75	62
sigN103	DDB0191897	DDB0230164	6	1 – 13//2 – 268	89	93	41
sigN107	DDB0191896	DDB0231563	6	1 – 13//2 – 268	111	93	35
sigN110	DDB0168566		2	1 – 13//2 – 250	110	87	33

70% of the amino acids of the encoded proteins are identical to SigN1 (Table 1). However, the identity among the proteins encoded by this second group of genes was about 95% (Fig 1B), which made them group together in the phylogenetic tree (Fig 2). These genes have been named sigN6, 7, 8, 9, 11, 12 and 14 (Group 2). The identity of the proteins encoded by the two groups of genes was very high in their N-terminal regions. However, there were some group-specific differences at the C-terminal region (Fig 1B). Genes DDB0217094 (sigN105) and DDB0168488 (sigN10) are located in the chromosomal region of group 1 and 2 genes, respectively. They have not been included in these groups because of their lower identity with the other genes, as shown in the phylogenetic tree (Fig 2). The identity of the hypothetical protein encoded by sigN105 with group 1 proteins was about 62% and that encoded by sigN10 showed an identity with the members of group 2 of about 64%.

Temporal expression of sigN *genes*

The cDNA isolated in the differential screening was used as a probe to analyze the developmental expression of these genes in WT and *srfA*- strains by Northern blot (Fig 3A). No expression was detected in proliferating cells (time 0 of development) or during the first hours of development. *sigN* expression was induced at 10 hours in WT cells and is maintained during the rest of development. SrfA mutant strains also showed induction of *sigN* expression at 12 hours of development but gene expression was lower than in WT cells, especially at later stages of development. The expression of *sigN* at the slug stage of development was also lower in the *srfA*- strains than in WT, in agreement with the results obtained in the subtractive method used for isolation of the gene.

The probe used to determine the differential expression of *sigN* covered 190 nt in the coding sequence of this gene. It is very likely that the probe can detect Group 1 and Group 2 mRNAs, due to the high identity that exists among the genes. Specific oligonucleotides were designed to study the expression of the two groups of genes independently, based on the differences in their C-terminal-coding regions. RT-PCR studies showed that expression of Group 1 genes was first detected at 2 hours of development with a rise at 12 hours, when the mound is completely formed (Fig 3B). Group 2 genes showed no expression until 10 hours of development with maximal induction obtained at 14 hours. In this case, the lower bands obtained in the RT-PCR reactions were sequenced and did not correspond to any specific amplification product.

Analyses of sigN *promoters*

The temporal pattern of expression of the *sigN* genes and their partial dependence on the SrfA transcription factor prompted the interest in the study of their promoter regions. The complete intergenic region upstream of each gene, up to the closest coding region, was considered putative promoter region in these studies. In some cases, genes are adjacent and in opposite orientation (For example, genes *sigN1* and *sigN3* in Fig 2A) and their putative promoter regions are complementary to each other. Clustal alignment studies were performed to find possible identities among putative promoter regions. The intergenic regions of Group 2 genes showed a high sequence

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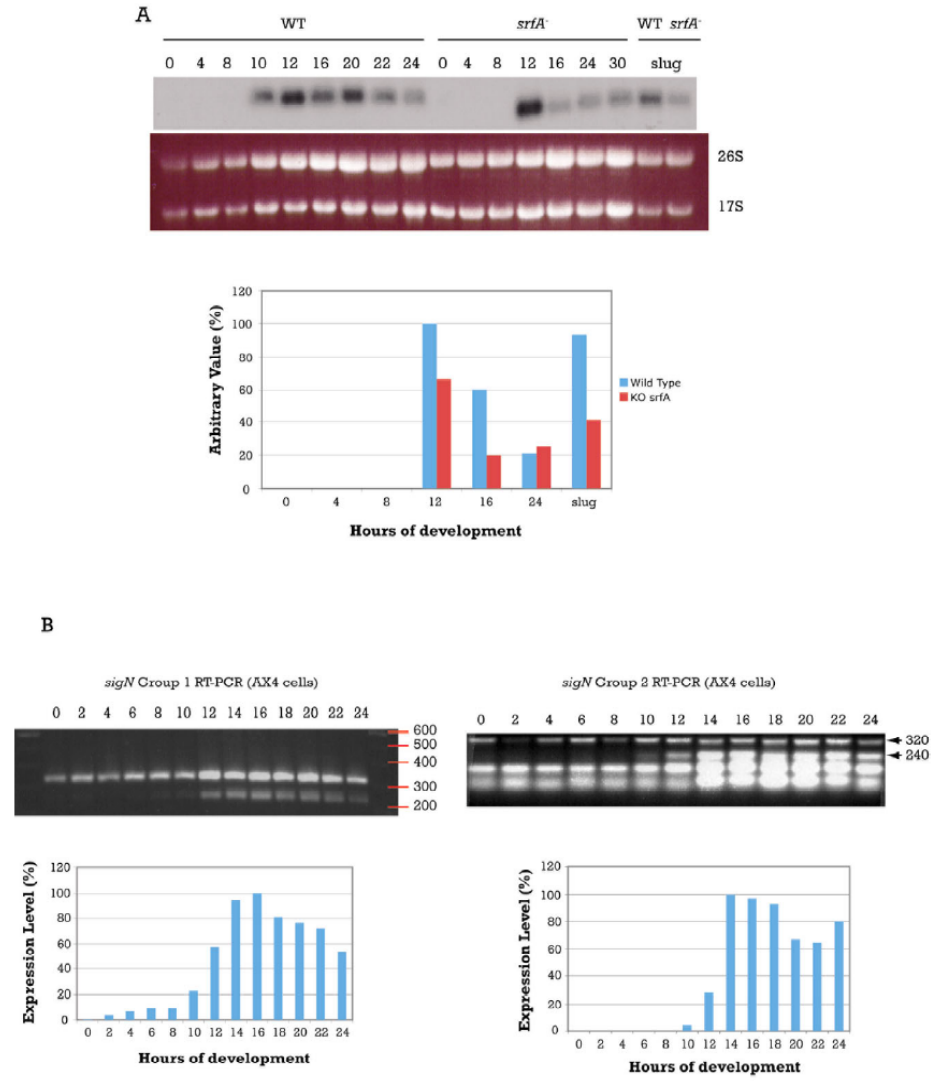

Figure 3

Expression pattern of *sigN* genes during multicellular development. A. RNAs from WT and *srfA*- strains were collected at the indicated hours (0–30) or at the slug stage of development (slug). The coding region of the *sigN1* gene was used as probe for hybridization. The mRNA detected migrated faster than the 18S rRNA. Ethidium bromide staining of the gel is shown in the lower panel. Hybridization signals were quantified by densitometry and normalized for the rRNA present in each lane (lower graphic). The value obtained for the 12 hours sample of the WT strain was considered 100% of expression. B. RNA was obtained from WT (AX4) strains, collected at the indicated hours of development. *SigN* genes expression was determined by RT-PCR using oligonucleotides specific for each group of genes. The sequence amplified for both groups was about 240 bp, and the control sequence, obtained by amplification of a region of the large mitochondrial ribosomal RNA, about 320 bp. Bands were quantified by densitometry, normalized in relation to the control and represented in a bar graphic, assigning 100% expression to the16 hours sample for Group 1 and 14 hours sample for Group 2.

identity (Fig 4A). The similarities of the putative promoter regions are indicated in the phylogenectic tree shown in Figure 4B. Promoter regions of the genes *sigN7* and *9* (and their complementary sequences *sigN12* and *14*) are more similar between them and slightly more distant from *sigN8* (complementary to *sigN11*) and *sigN6*. Putative promoter regions of adjacent genes, that are inverted in relation to each other, also shown significant identity among them indicating their palindromic structure, as schematically shown in Figure 4C. These data indicate that the genes in this group could have derived by successive duplication of a tandem of two inverted *sigN* genes.

Intergenic regions of the Group 1 genes *sigN1-sigN3* and *sigN2-sigN4* are very similar between them but do not have the palindromic structure described for Group 2 genes. Therefore, the putative promoter region of the *sigN1* gene is very similar to that of *sigN2* and the same hold true for *sigN3* and *sigN4* regions. However, the *sigN1* promoter region does not show similarity to that of the contiguous *sigN3* gene. Similarly, the *sigN2* putative promoter region does not show similarity to that of *sigN4*. These regions do not show similarity with the sigN5 and sigN25 putative promoter regions, either.

The comparison of the nucleotide sequence of the putative promoter regions of Group2 genes detected the existence of four repetition of a conserved G-rich sequence (boxed in Fig 4A), also present in Group 1 promoters. These sequences are very similar to the GBF response element: (T/G)G (T/G)G (T/G)G (T/G) [32]. Group 2 promoter regions contain two pairs of closely locate GBF-binding sites, labelled as regions A and B in Figures 4A and 4C, that could regulate the expression of the contiguous opposite genes.

The functionality of the putative promoter regions was tested using reporter vectors where a *lacZ* gene coding for unstable β-galactosidase was placed under the transcriptional control of *sigN* promoters. These assays also allowed determining the spatial pattern of expression of these genes during development. The high similarity of these genes made very difficult the use of *in situ* hybridization to study the expression of specific genes of this family during development. These studies were focused on putative promoter regions of one Group 1 gene (*sigN2*) and one of the Group 2 genes (*sigN9*) given the similarity observed between the promoters of each group. Staining of the structures transformed with the reporter vectors indicate that both putative promoter regions direct gene expression in the prestalk region of developing structures (Fig 5). Promoter of the *sigN2* gene showed weaker activity in these assays but *lacZ* expression was first observed in disperse cells in the mound stage of development. Later on, prestalk cells placed at the tip of the structure showed *lacZ* expression in first finger and early culminant structures (Fig 5A). At the slug stage promoter activity was detected in cells dispersed through the structure, with a pattern similar to that of Anterior Like Cells (Fig 5A). The promoter of the *sigN9* gene also directed expression in dispersed cells in mounds and in prestalk cells at the tip of first finger and early culminant structures and in dispersed cells in the slugs (Fig 5B). In addition, *sigN9* promoter also directed lacZ expression in the cells that migrate from the tip to form the stalk during culmination and in stalk cells of the fruiting body.

Function of sigN *genes*

Over-expression and under-expression of *sigN* genes was intended to study the function of these genes. Over-expression was performed by cloning the gene *sigN4* under the control of the actin 15 promoter. Western blot analyses of vegetative cells using antibodies specific for Group 1 proteins indicated the expression of a 12 kDa protein in the over-expressing strains that was not detected in the WT strain (Fig 6A). No difference in phenotype was found between the WT and the over-expression strains. Downregulation was initially approached by the expression of interfering RNA. Two copies of *sigN3* exon 2 were placed in an inverted orientation, separated by a stuffer sequence, under the control of an actin 15 promoter. The great identity in the N-terminal-coding region of these genes, could make possible that the iRNA fragments generated from this construct would interfere with the expression of all *sigN* genes. Strains expressing this iRNA construct showed a strong reduction in the expression of sigN RNAs (Fig 6B) but did not show any difference in their phenotype in relation to WT strains. Mutant strains were also obtained where all *sigN* genes of Group1, Group2 or both groups were deleted by homologous recombination. Deleted regions are indicated in Figure 1A. RT-PCR analysis was used to corroborate the absence of *sigN* expression in these strains (Fig 6C–E). However, neither of the mutant strains did show any difference in growth or development in comparison to the WT strain.

Many proteins are secreted during *Dictyostelium* development to coordinate morphogenesis and differentiation. Some of these proteins have low molecular weight and present post-translational modifications, like glycosylations. Examples of these molecules are PSF, CMF, countin factor and SDF, as mentioned in the Introduction section. The small size of SigN proteins, the existence of hypothetical glycosylation sites and the predicted presence of a cysteine-knot structure and a putative transmembrane region would be in agreement with an extra-cellular function for these proteins. To test this hypothesis, specific antibodies were raised against the proteins of each group. Immunocytochemical analysis using these antibodies detected staining in small vesicles present in a fraction of

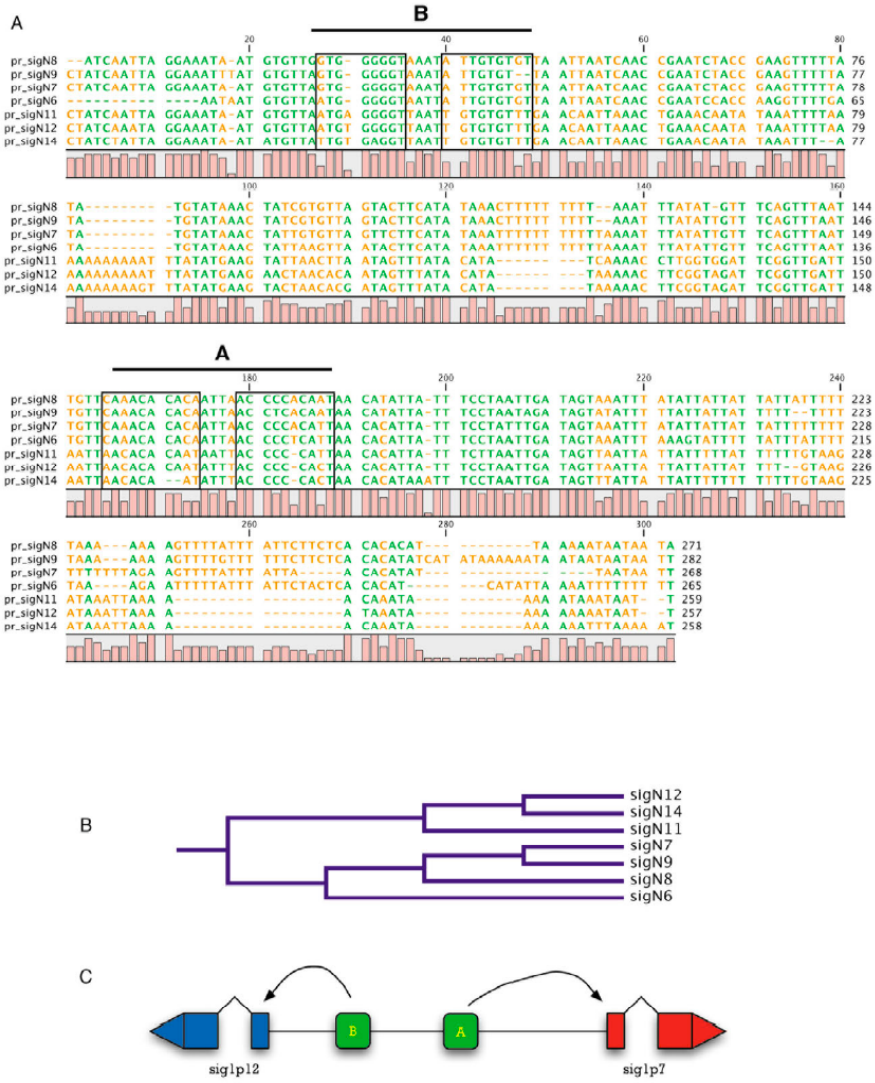

Figure 4

Similarity of the putative promoters regions of *sigN* Group 2 genes. A. The sequences of the putative promoters of *sigN* Group 2 genes, considered as the upstream regions from the initiation codon to the closest gene-coding region, were compared and the alignment of the regions containing putative regulatory sites is shown. The boxes in the figure indicate the possible binding sites for the transcription factor GBF (G-box Binding Factor). Pairs of contiguous binding sites have been grouped and labelled as regulatory regions A and B. Identical nucleotides are indicated in green. The level of identity between nucleotides of the different genes is represented as bars (conservation). B. Phylogenetic tree calculated from the nucleotides alignments shown in panel A using the Clustal W program. C. Schematic representation of the location of conserved, potential regulatory regions (A and B) present in the intergenic region between each pair of inverted genes, represented as rounded boxes. Coding regions are represented as boxes and intron and untranslated regions as lines. The direction of transcription of each gene is represented by arrow heads.

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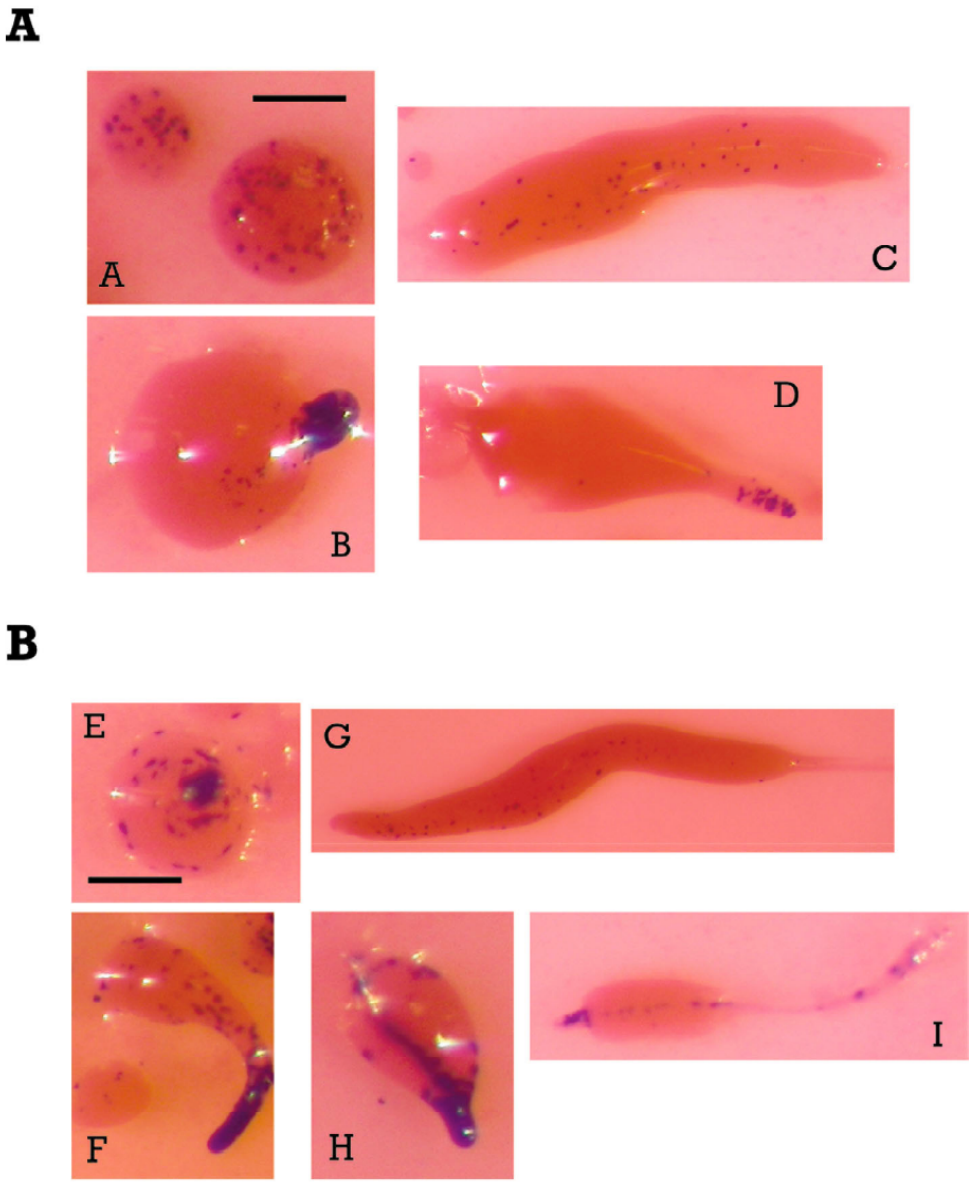

Figure 5
Cell-type specific activity of *sigN* gene promoters. A. AX-4 cells were transfected with a reporter vector driving *lacZ* expression under the control of the Group 1 *sigN2* promoter. β-galactosidase activity, detected by X-gal staining, is shown in structures at mound (A), finger (B), slug (C) and early culminant (D) structures (Bar, 0,1 mm). B. A reporter vector where lacZ was placed under the transcriptional control of the Group 2 *sigN9* promoter was transfected in AX-4 cells. X-gal staining is shown for mound (E), finger (F), slug (G), early-culminant (H) and culminant (I) structures (Bar, 0,1 mm).

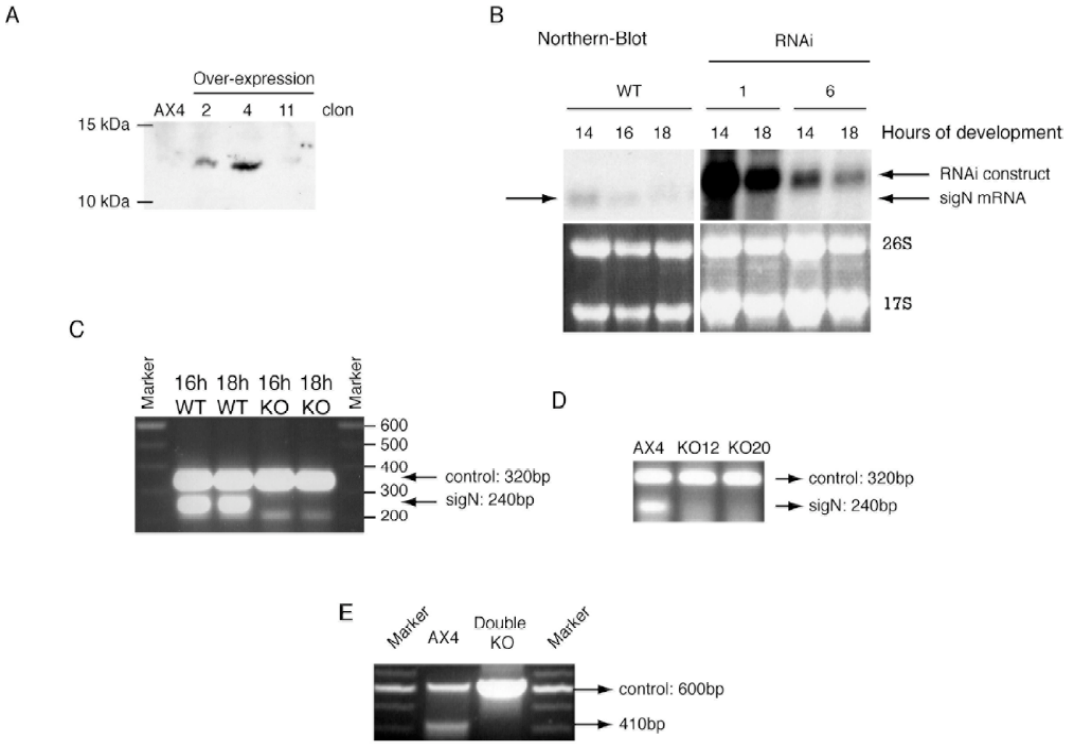

Figure 6
Characterization of strains where *sigN* genes have been over-expressed, repressed by the use of interfering RNA or deleted. A. Total protein extracts were obtained from WT (AX4) and three strains (2, 4, 11) transfected with a plasmid vector for *sigN4* over-expression. Group 1 antibodies were used for Western-Blot. The migration of the 10 and 15 kDa protein markers is shown on the left. B. RNA was obtained from WT and two different RNAi clones that expressed an interfering RNA based on exon 2 of *sigN3* (1 and 6 clones) after 14–18 hours of development on Nitrocellulose filters. Arrows indicate the position of the endogenous mRNA (*sigN* mRNA) and of the RNAi transcribed from the vector (RNAi construct). Ethidium bromide staining of the gel is shown in the lower panel. C. RNA from WT (AX4) and Group 1 KO strains, harvested at the indicated hours of development (16–18 h) was analyzed by RT-PCR for expression of *sigN* Group 1 genes. The control band obtained by amplification of a region of the large mitochondrial ribosomal RNA (control: 320 bp long) and that corresponding to *sigN* Group 1 mRNA (*sigN*:240 bp) are indicated by arrows at the right. D. RNA was obtained from WT (AX4) and two different Group 2 KO strains (KO12, KO20) after 16 hours of development. Expression of *sigN* Group 2 genes was analyzed by RT-PCR. The migration of the control band (control: 320 bp) and that corresponding to *sigN* Group 2 mRNA (*sigN*: 240 bp) is indicated at the right. E. DNA from WT (AX4) and a Group 1 and Group 2 Double KO strain (Double KO) was analyzed by PCR for the presence of for *sigN* Group 1 genes. Migration of the amplified fragments for a control gene (control: 600 bp) and *sigN* Group 1 genes (410 bp) is indicated at the right.

the cells obtained after dispersion of the cells from mound structures obtained after 14 hours of development (Fig 7). Staining was not observed in vegetative cells, using immune or preimmune serum, or in mound cells using pre-immune serum (Fig 7). Staining in vesicles would be in agreement with the hypothesis that Group 1 and Group 2 proteins are secreted to the extra-cellular medium.

To further study the possible function of these proteins, antibodies were added in vivo to developing cells to try interfering some of the developmental processes. Pre-immune antiserum was used as control. Specific antibodies against Group 1 proteins did not show any obvious interference with the developmental process in comparison with pre-immune and phosphate-based buffer (PDF) controls.

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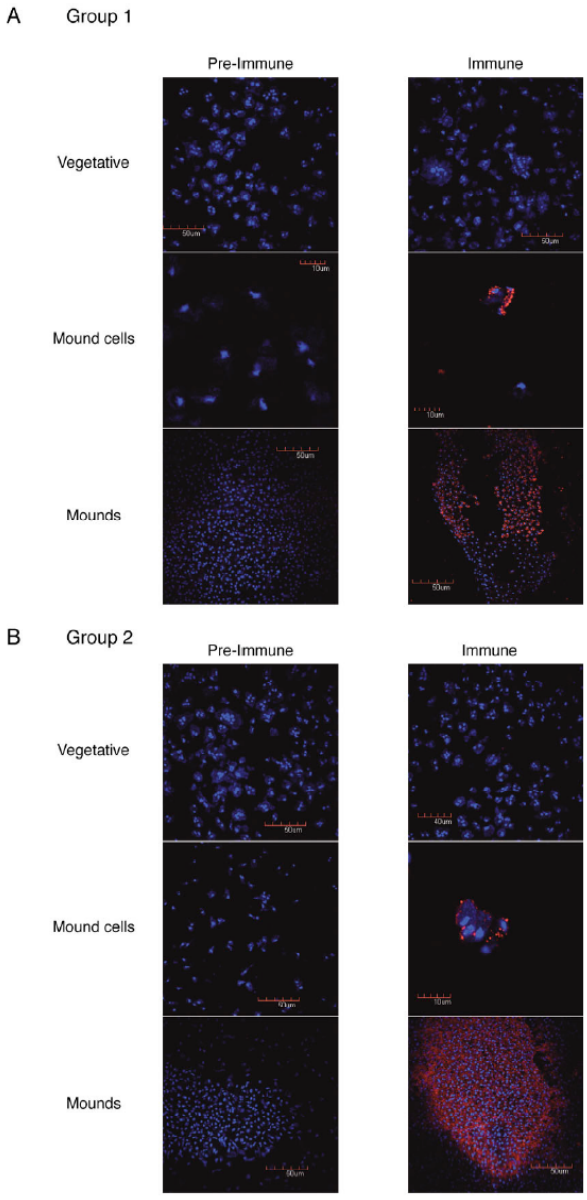

Figure 7
Immunocyotchemical analyses of SigN expression. Vegetative cells (vegetative), cells obtained by dispersion of mound structures developed for 14 hours (Mound cells) or mound structures (Mounds) were fixed and stained with preimmune serum (Pre-immune) or serum raised against specific peptides (Immune) corresponding to Group 1 (upper panels) or Group 2 (lower panels) genes Alexa 568-conjugated anti-rabbit serum was used as secondary antibody. DAPI was used for nuclear staining.

The addition of antibodies specific for the Group 2 proteins produced some differences, in comparison to pre-immune or PDF controls. Mounds were formed two hours earlier in the presence of Group2-specific antibodies. From there on, mounds began to disaggregate and, at 15 hours of development, when a finger is normally formed, no visible structure remained in the filters (Fig 8). At 15 hours of development the filter treated with the antibody was divided in two parts. One of them was incubated for 10 more hours with a PDF phosphate-based buffer, and the other with antibody added to fresh medium. The part incubated in PDF developed in a usual way, forming new mounds. The part of the filter that continued in the presence of the antibody was unable to form any structure (Fig 8). These results make us suggest that *sigN* Group 2 genes could be involved in the aggregation process, contributing to maintain the coherence of the multi-cellular structure.

Discussion

The study of the gene *sigN1*, partially dependent on the transcription factor SrfA for expression, has uncovered the existence of a family of genes coding for very similar small proteins in *D. discoideum*. The family is large with, at least, 96 genes, although the present study has been centred on 13 closely related genes located in two regions of chromosome 2. These genes have in common their structure and developmental pattern of expression, in addition to the high similarity of their nucleotide sequences. These genes have been divided in two groups based on the sequence of a small divergent region that code for the C-terminal region of the protein, and their chromosomal location. Phylogenetic analyses of the coding and intergenic regions suggest that the genes of each group might have originated by duplication of an original tandem of two genes, oriented in opposite directions.

All the genes are constituted by an exon, containing a 13 nt long open reading frame, an intron and a second exon, coding for the rest or the protein. The same structure is share by most of the 96 similar genes mentioned above. The conserved structure of these genes might be indicative of their common evolutionary origin.

Northern and RT-PCR studies have shown that Group1 and Group2 *sigN* genes expression is induced at 10–12 hours of multi-cellular development. Analyses of the promoter regions of one gene of each group indicate that *sigN* genes are expressed in the prestalk region. The sequence of the putative promoter regions, very similar among Group 2 genes, contain several copies of a conserved G/C rich motif similar to the GBF transcription factor binding site [32]. GBF expression is developmentally regulated and necessary for the expression of numerous prestalk and prespore genes [10]. Microarray analysis of GBF-dependent genes identified *sigN12* as one of them [18], in agreement with the presence of putative GBF-binding sites in the promoter region. Two other *sigN* related genes, DDB0230164 (*sigN103*) and DDB0231563 (*sigN107*), coding for proteins that are 41% and 35% identical to SigN1, respectively, are also dependent on GBF for their expression The developmental pattern of expression of these genes was also very similar to that of *sigN* genes. Besides, Microarray and in situ hybridization analyses of the expression of these genes have shown that these three GBF-dependent genes are expressed in the prestalk region, including *sigN12* [18,33]. These data are in agreement with the results presented in this article and suggest that, since all these genes are very similar in sequence and present similar temporal and spatial patterns of expression, might accomplish similar, and perhaps redundant, biological functions.

The study of the function of *sigN* genes was approached by their over-expression, under-expression and by deletion. However, even the deletion of the 13 Group 1 and Group 2 genes seemed to have no effect on development. These data could be explained by the redundant functions of this family of genes, as previously suggested on the bases of their structure and expression.

Only the addition of specific antibodies raised against Group 2 *sigN* genes had some effect on development, inducing disaggregration of mound structures. This effect was specific since antibodies raised against Group 1 peptides or pre-immune serum had no effect on this process. With the exception of the *sigN4* over-expressing strain, the antibodies raised against SigN peptides did not recognize these proteins in Western blot, which precluded further studies on their specificity. With this caveat in mind, the simplest explanation for these results obtained would be that Group 2 SigN proteins could participate in cell-cell adhesion and that the presence of the antibody could block interactions of SigN with other proteins, required for cell adhesion. As mentioned in the Results section, many proteins containing cysteine knot domains are extracellular, which would be in agreement with the function proposed for SigN Group 2 proteins. Antibodies raised against Group 1 and Group 2 proteins stained small vesicles in cells at the mound stage or development, which would be also in agreement with their proposed secretion. The expression pattern was similar for both antibodies, as expected from the similar structural domains predicted for the proteins of both groups.

According to the hypothesis suggested above, *sigN* Group 2 mutants would be expected to show defective aggregation, which was not the case. As mentioned above, functional redundancy between *sigN* genes could explain these results since the sig *N* Group 2 mutants could still maintain cell adhesion through the interaction of other related

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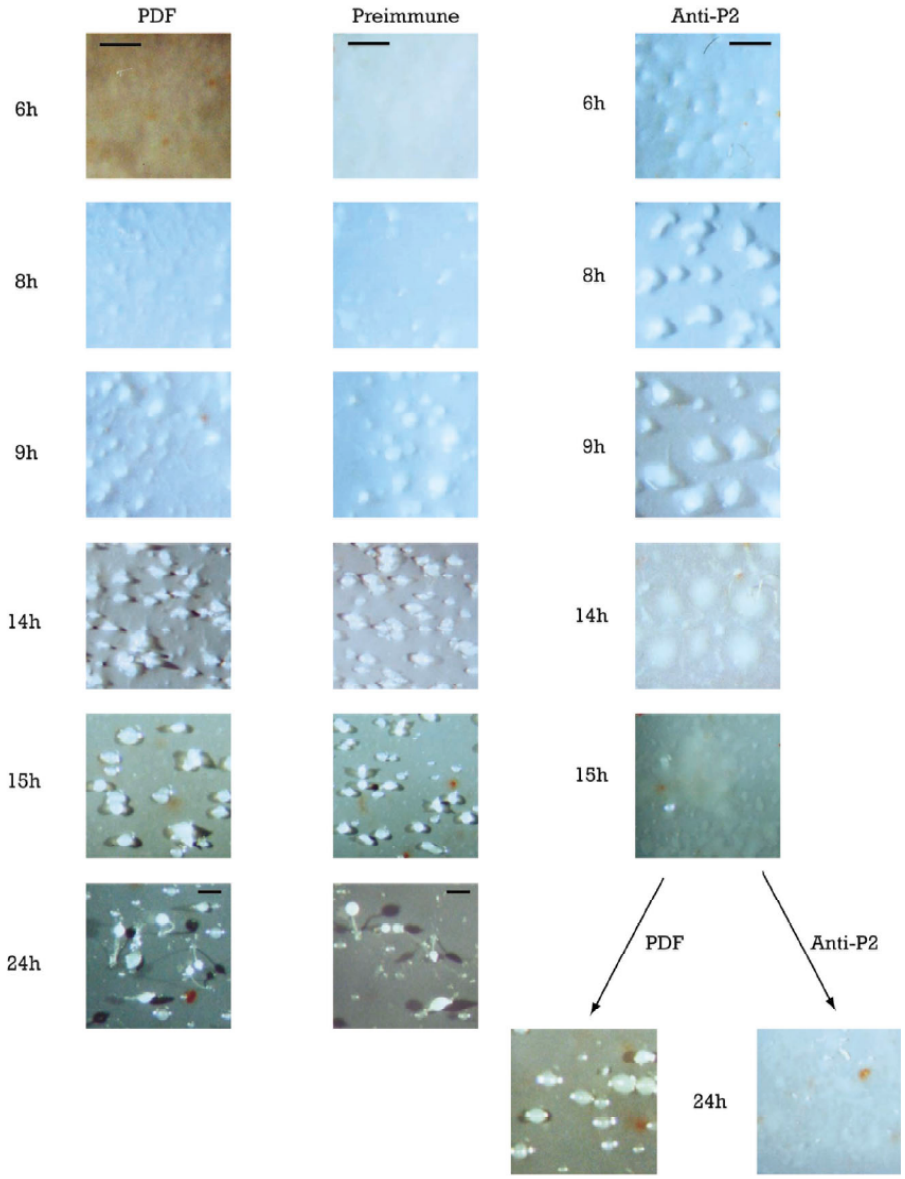

Figure 8
Development in the presence of antibodies specific for Group 2 *sigN* proteins. AX4 cells were placed on nitrocellulose filters to induce multicellular development. Filters were placed over pads soaked on phosphate buffer alone (PDF) or containing preimmune serum (preimmune) or a serum raised against a Group 2-specific peptide (Anti-P2). At 15 hours of development, the antiserum filter was divided in two parts. One half was placed on a new pad soaked in PDF alone (PDF) and the other on a pad soaked in PDF containing Group 2 antiserum (Anti-P2), as shown in the lower part of the right-hand panels. Pictures were taken at the times of development indicated at the left of the panels with a stereomicroscope (Bar, 0,15 mm).

6. Wong LM, Siu CH: **Cloning of cDNA for the contact site A glycoprotein of Dictostelium discoideum.** *Proc Natl Acad Sci USA* 1986, **83**:4248-4252.
7. Dynes JL, Clark AM, Shaulsky G, Kuspa A, Loomis WF, Firtel RA: **LagC is required for cell-cell interactions that are essential for cell-type differentiation in Dictyostelium.** *Genes Devel* 1994, **8**:948-958.
8. Siu CH, Brar P, Fritz IB: **Inhibition of cell-cell adhesion and morphogenesis of Dictyostelium by carnitine.** *J Cell Physiol* 1992, **152**:157-165.
9. Kamboj RK, Gariepy J, Siu CH: **Identification of an octapeptide involved in homophilic interaction of the cell adhesion molecule gp80 of Dictyostelium discoideum.** *Cell* 1989, **59**:615-625.
10. Schnitzler GR, Fischer WH, Firtel RA: **Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in Dictyostelium.** *Genes Devel* 1994, **8**:502-514.
11. Clow PA, Chen TLL, Chisholm RL, McNally JG: **Three-dimensional in vivo analysis of Dictyostelium mounds reveals directional sorting of prestalk cells and defines a role for the myosin II regulatory light chain in prestalk cell sorting and tip protrusion.** *Development* 2000, **127**:2715-2728.
12. Berks M, Kay RR: **Combinatorial control of cell differentiation by cAMP and DIF-1 during development of Dictyostelium discoideum.** *Development* 1990, **110**:977-984.
13. Roisin-Bouffay C, Jang W, Caprette DR, Gomer RH: **A precise group size in Dictyostelium is generated by a cell- counting factor modulating cell-cell adhesion.** *Mol Cell* 2000, **6**:953-959.
14. Varney TR, Casademunt E, Ho HN, Petty C, Dolman J, Blumberg DD: **A novel Dictyostelium gene encoding multiple repeats of adhesion inhibitor-like domains has effects on cell-cell and cell-substrate adhesion.** *Dev Biol* 2002, **243**:226-248.
15. Anjard C, Loomis W: **Peptide signaling during terminal differentiation of Dictyostelium.** *Proc Natl Acad Sci U S A* 2005, **102**:7607-7611.
16. Iranfar N Fuller, D. and Loomis, W.F.: **Gene regulation during early development of Dictyostelium using genome-wide expression analyses.** *Eukaryotic Cell* 2003, **2**:664-670.
17. Williams JG: **Transcriptional regulation of Dictyostelium pattern formation.** *EMBO Rep* 2006, **7**:694-698.
18. Iranfar N, Fuller D, Loomis W: **Transcriptional regulation of post-aggregation genes in Dictyostelium by a feed-forward loop involving GBF and lagC.** *Dev Biol* 2006, **290**:460-469.
19. Escalante R, Sastre L: **A serum response factor homolog is required for spore differentiation in Dictyostelium.** *Development* 1998, **125**:3801-3808.
20. Escalante R, Vicente JJ, Moreno N, Sastre L: **The MADS-box gene srfA is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of Dictyostelium development.** *Dev Biol* 2001, **235**:314-329.
21. Escalante R Moreno, N., and Sastre, L.: **Dictyostelium discoideum developmentally regulated genes whose expression is dependent on the MADS-box transcription factor SrfA.** *Eukaryotic Cell* 2003, **2**:1327-1335.
22. Escalante R Iranfar, N., Sastre, L., Loomis, W.F.: **Identification of genes dependent on the MADS-box transcription factor SrfA in Dictyostelium development.** *Eukaryotic Cell* 2004, **3**:564-566.
23. Sussman M: **Cultivation and synchronous morphogenesis of Dictyostelium under controlled experimental conditions.** In *Methods in Cell Biology Volume 28*. Edited by: Spudich JA. Orlando, FL, Ac. Press; 1987:9-29.
24. Kuspa A, Loomis WF: **Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA.** *Proc Natl Acad Sci USA* 1992, **89**:8803-8807.
25. Adachi H, Hasebe T, Yoshinaga K, Ohta T, Sutoh K: **Isolation of Dictyostelium discoideum cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker.** *Biochem Biophys Res Commun* 1994, **205**:1808-1814.
26. Shaulsky G, Loomis WF: **Cell type regulation in response to expression of ricin-A in Dictyostelium.** *Dev Biol* 1993, **160**:85-98.
27. Detterbeck S, Morandini P, Wetterauer B, Bachmair A, Fischer K, MacWilliams HK: **The 'prespore-like cells' of Dictyostelium have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters.** *Development* 1994, **120**:2847-2855.
28. Escalante R, Sastre L: **Investigating gene expression: In situ hybridization and reporter genes.** In *Dictyostelium discoideum protocols Volume 346*. Edited by: Eichinger L and Rivero F. Totowa, NJ, Humana Press; 2006:230-247.
29. Clayton CE, Estevez AM, Hartmann A, Alibu VP, Field M, Horn D: **Down-regulating gene expression by RNA interference inTrypanosoma brucei.** *Methods Mol Biol* 2005, **309**:39-60.
30. Manstein DJ, Schuster HP, Morandini P, Hunt DM: **Cloning vectors for the production of proteins in Dictyostelium discoideum.** *Gene* 1995, **162**:129-134.
31. Escalante R Yamada, Y., Cotter, D., Sastre, L., and Sameshima, M.: **The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during Dictyostelium sporulation.** *Mechanisms of Development* 2004, **121**:51-56.
32. Hjorth AL, Pears C, Williams JG, Firtel RA: **A developmentally regulated trans-acting factor recognizes dissimilar G/C-rich elements controlling a class of cAMP-inducible Dictyostelium genes.** *Genes Devel* 1990, **4**:419-432.
33. Maeda M Sakamoto, H., Iranfar, N., Fuller, D., Maruo, T., Ogihara, S., Morio, T., Urushihara, H., Tanaka, Y., and Loomis, W.F.: **Changing patterns of gene expression in prestalk cell subtypes of Dictyostelium recognized by in situ hybridization with genes from microarray analyses.** *Eukaryotic Cell* 2003, **2**:627-637.
34. Harloff C, Gerisch G, Noegel AA: **Selective elimination of the contact site A protein of Dictyostelium discoideum by gene disruption.** *Genes Devel* 1989, **3**:2011-2019.
35. Loomis WF: **Cell-cell adhesion in Dictyostelium discoideum.** *Dev Genet* 1988, **9**:549-559.
36. Freeze H, Loomis W: **The isolation and characterization of a component of the surface sheath of Dictyostelium discoideum.** *J Biol Chem* 1977, **252**:820-824.

Artículo 2: *The dual-specificity protein phosphatase MkpB, homologous to mammalian MKP phophatases, is required for D. discoideum post-aggregative development and cisplatin response*

Las proteínas fosfatasas de especificidad dual participan en vías de transducción de señales inactivando a las proteínas quinasas activadas por mitógeno (MAP quinasas). Dichas vías de señalización son de crucial importancia en la regulación de numerosos procesos biológicos, incluyendo proliferación celular, diferenciación y desarrollo. La ameba social *Dictyostelium discoideum* tiene 14 genes que codifican proteínas que contienen regiones similares a los dominios presentes en las fosfatasas de especificidad dual. Uno de esos genes, *mkpB* adicionalmente presenta, en la región N-terminal, un dominio llamado Rodanasa, característico de las MAP quinasas–fosfatasas animales. Las células que sobre-expresan este gen muestran mayor actividad fosfatasa. *mkpB* se expresa en *D. discoideum* durante el crecimiento pero el mayor nivel de expresión se observa a las 12 horas de desarrollo. Aunque es expresado en todas las células de las estructuras en desarrollo, el ARNm de *mkpB* está enriquecido en las células denominadas *"Anterior like cells"*. Las células que expresan una forma mutante catalíticamente inactiva de MkpB crecen y agregan como la cepa silvestre pero sufren defectos en los estadios posteriores a la agregación. Además, la expresión de genes específicos de tipo celular se encuentra retrasada, indicando que esta proteína juega un papel importante en diferenciación celular y desarrollo. Las células que expresan la forma mutante catalíticamente inactiva de MkpB muestran mayor sensibilidad a cisplatino, mientras que las que sobre-expresan la forma silvestre de MkpB o MkpA, así como células mutantes para el gen de la MAP quinasa *erkB,* son más resistentes a la aplicación de cisplatino como droga quimioterapeútica. También se ha observado una relación similar entre la actividad de MKP y la sensibilidad a cisplatino en células tumorales.

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The dual-specificity protein phosphatase MkpB, homologous to mammalian MKP phosphatases, is required for *D. discoideum* post-aggregative development and cisplatin response ☆

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ABSTRACT

Dual-specificity protein phosphatases participate in signal transduction pathways inactivating mitogen-activated protein kinases (MAP kinases). These signaling pathways are of critical importance in the regulation of numerous biological processes, including cell proliferation, differentiation and development. The social ameba *Dictyostelium discoideum* harbors 14 genes coding for proteins containing regions very similar to the dual-specificity protein phosphatase domain. One of these genes, *mkpB*, additionally codes for a region similar to the Rhodanase domain, characteristic of animal MAP kinase-phosphatases, in its N-terminal region. Cells that over-express this gene show increased protein phosphatase activity. *mkpB* is expressed in *D. discoideum* ameba at growth but it is greatly induced at 12 h of multicellular development. Although it is expressed in all the cells of developmental structures, *mkpB* mRNA is enriched in cells with a distribution typical of anterior-like cells. Cells that express a catalytically inactive mutant of MkpB grow and aggregate like wild-type cells but show a greatly impaired post-aggregative development. In addition, the expression of cell-type specific genes is very delayed, indicating that this protein plays an important role in cell differentiation and development. Cells expressing the MkpB catalytically inactive mutant show increased sensitivity to cisplatin, while cells over-expressing wild type MkpB, or MkpA, proteins or mutated in the MAP kinase *erkB* gene are more resistant to this chemotherapeutic drug, as also shown in human tumor cells.

1. Introduction

Mitogen-activated protein kinases (MAPK) are important intracellular signal transducer proteins involved in the regulation of cell proliferation, differentiation, migration and apoptosis (reviewed in Johnson and Lapadat, 2002; Pearson et al., 2001). These proteins are activated by phosphorylation on both the threonine and tyrosine residues of the consensus T-X-Y domain in response to extracellular signals (reviewed in Qi and Elion, 2005). MAP kinases activation is a reversible process and the period of time that the proteins remain activated is an important determinant of the biological response (Marshall, 1995). Inactivation is produced by dephosphorylation of either the threonine or the tyrosine residues. Therefore, this reaction can be catalyzed by threonine- or tyrosine-protein phosphatases but an important group of enzymes that mediate this reaction are the dual-specificity phosphatases (DUSP) that dephosphorylate both residues (reviewed in Dickinson and Keyse, 2006; Patterson et al., 2009).

Dual-specificity protein phosphatases constitute themselves a large family of proteins in mammals, characterized by the presence of a highly conserved C-terminal catalytic domain. Although all the enzymes of the family can inactivate MAP kinases, the largest group of mammalian phosphatases involved in MAPK signaling regulation belongs to the subgroup of the MAPK phosphatases (MKP) (Keyse, 2008). This group of 11 human proteins share an N-terminal domain characterized by the presence of two regions of similarity to the cdc25 protein phosphatase catalytic domain (Keyse and Ginsburg, 1993). This domain, that is important for MAPK recognition and binding, is also present in the Rhodanase family of sulphotransferases and has been named Rhodanase domain.

One of the organisms where the MAPK regulatory pathway has been shown to be important is the social ameba *Dictyostelium discoideum*, that constitutes one of the simplest models for the study of cell motility, differentiation and multicellular development (for a recent review see Annesley and Fisher, 2009)

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This organism lives in forestall soils as individual ameba, feeding on bacteria. However, when food is exhausted ameba aggregate together and initiate a multicellular developmental process leading to the formation of a fruiting body. This structure is composed of a basal disk, a stalk and, on top of it, a sorus where most of the ameba differentiate into spores. This is an adaptive response since spores are resistant to adverse conditions for several weeks but germinate to give rise to new ameba in a favorable environment.

Analysis of the nucleotide sequence of the *D. discoideum* genome (Eichinger et al., 2005) has shown that this organism harbor two MAPK-encoding genes, *erkA* and *erkB*. *erkA* codes for a protein (Erk1) more similar to the ubiquitous ERK subfamily. Cells where this gene has been interrupted form small aggregates most of which get stalled at this developmental stage (Sobko et al., 2002).

The *erkB* gene codes for a protein (Erk2) that is more similar to the ERK7 subfamily of MAP kinases. Mutagenesis studies have shown that this gene is required for cAMP synthesis in response to extracellular cAMP. In *D. discoideum* aggregation is produced by chemotaxis towards extracellular cAMP. This molecule is initially secreted at discrete aggregation centers and diffuses towards neighboring cells. Later on, each cell of the aggregation field that detects an increase in cAMP concentration responds by migrating towards increasing cAMP concentrations. In addition, each cell secretes cAMP so that the signals gets amplified and moves through the field of cells as cAMP waves (van Haastert and Devreotes, 2004). Cells moving towards the aggregation centers also adhere to each other, forming cell streams. Therefore, *erkB* mutant cells, that are defective in cAMP secretion, show greatly impaired cell aggregation (Segall et al., 1995). Actually, Erk2 has been proposed to be a component of an oscillatory circuit that regulates cAMP synthesis during aggregation (Maeda et al., 2004).

A protein phosphatase with a DUSP phosphatase domain and the capacity to dephosphorylate Erk2 has been described (Rodriguez et al., 2008). This enzyme, named MPL1, is induced at 5 h of development, at the initiation of the aggregation process. The *mpl1* gene was ablated by homologous recombination and the mutant cells showed persistent Erk2 phosphorylation, aberrant patterns of cAMP production, defective aggregation and streaming (Rodriguez et al., 2008). A second gene coding for a DUSP-domain containing protein, *mkpA*, has been interrupted by insertional mutagenesis and the mutant cells were defective in cAMP wave formation and aggregation (Sawai et al., 2007). These data, and those described above for Erk2, highlight the importance of MAPK signaling during the aggregation of *D. discoideum* cells.

However, the picture is not complete because the analysis of the *D. discoideum* genome indicates the existence of 14 genes coding for proteins that contain DUSP phosphatase domains, including MkpA and MPL1. It will be of interest to determine the possible function of all these genes and their possible interactions. This article approaches the study of one of these genes, named *mkpB*. The encoded protein is unique among *D. discoideum* DUSP-domain containing proteins because in its N-terminal region contains a Rhodanase domain similar to that of the mammalian MKP subfamily of dual-specificity phosphatases. The phosphatase domain of MkpB is also more similar to those of the mammalian MKP1 and MKP4 proteins than most of the other *D. discoideum* proteins, suggesting that this protein could play a relevant role in Erk1 or/and Erk2 activity regulation. Unexpectedly, *mkpB* expression is induced by 12 h of multicellular development, after aggregation is completed. In addition, over-expression of a catalytically inactive mutant protein did not affect cell aggregation but impaired post-aggregative development. These data suggest that MkpB plays an important role during *D. discoideum* development regulation, acting at later stages than MkpA and MPL1. Additionally, analyses of cisplatin sensitivity of cells that express MkpB, the catalytically inactive mutant of MkpB, MkpA and ErkB mutant cells indicated that these signaling pathways are involved in the response to this anti-carcinogenic drug.

2. Methods

2.1. Cell culture, transformation and development

D. discoideum AX4 cells were cultured axenically in HL-5 media. Transformation by electroporation was performed as described by Pang et al. (1999). Transformed cells were selected by treatment with neomycin (G418) or blasticidine. For development on plastic plates 10^6 cells were collected by centrifugation, resuspended in 1 ml of phosphate-based PDF buffer and loaded on 6-well culture dishes (Multiwell, 6 well, FALCON, Becton Dickinson Labware, Franklin Lakes, NJ, USA). For development on filters, 2×10^7 cells were centrifuged, resuspended on PDF buffer and deposited on Nitrocellulose filters (Millipore) over PDF soaked absorbant pads (Millipore). Cells transformed with promoter reporter vectors were developed on Nitrocellulose filters and the β-galactosidase activity determined by X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) hydrolysis as previously described (Escalante and Sastre, 2006).

2.2. Generation of expression and reporter vectors

The coding region of the *mkpA* and *mpkB* (DDB0238328) genes was amplified by PCR using the oligonucleotides GGGGATCCACTCCACCATCAACTCCTAGAGG and GGGAATTCACCAACACCACCTTCACCACC for *mkpA* and GGGGATCCCAAGATATAGGATCTAGTAAG and GGGAATTCGGATTGAGGTAATT CAATTGGTG for *mkpB*. The fragments generated were cloned in the pGEMT-Easy plasmid vector (Promega), sequenced in both strands and transferred to the pDV-CTAP-CGFP expression vector (Meima et al., 2007) using the BamHI and EcoRI restriction sites. A catalytically inactive form of MkpB was generated by in vitro mutagenesis according to Zheng et al. (2004). The oligonucleotides GAGTTTTAATACACGCTGCAATGGGTATTAGTAGATC and CCCATTGCAGCGTGTATTAAAACTCTACCACCTTG were used for mutagenesis. *mkpB* promoter region, covering from the closest upstream gene (*alyD-2*)(nucleotide-1253 from the *mkpB* initiation codon) to nucleotide 45 of the *mkpB* coding region, was amplified by PCR using the primers GGTCTAGACCATAGCATGGGTTACTACAAC and GGACATCTTGGTAGTTCTTCTGGTTGTATATTAAC. The amplified fragments were cloned in the pGEMT-Easy vector, sequenced in both strands and transferred to the pDDGal-17 vector (Harwood and Drury, 1990), using the XbaI and BglII restriction sites.

2.3. Northern blot analysis

RNA was obtained from 2×10^7 cells collected from axenic cultures or from structures at different developmental stages on Nitrocellulose filters using the Trizol reagent (GIBCO-BRL). RNA electrophoresis, transfer to filter and hybridization were carried out as previously described (Sambrook et al., 1989). DNA probes were obtained by PCR from *D. discoideum* DNA using oligonucleotides designed from nucleotide sequences obtained at the Dicty Base (http://www.dictybase.org) and labeled using the Ready-to-Go DNA labeling Beads (-dCTP)(GE Healthcare).

2.4. DNA nucleotide sequence analysis

D. discoideum genes coding for proteins containing a DUSP phosphatase homology domain were identified at the Dicty Base using the amino acid sequence of the human MKP1 phosphatase domain as query and the on line BLASTP program. Amino acid sequences were aligned using the Clustal W algorithm of the MacVector program (MacVector Inc., Cary, NC, USA). Phylogenetic trees were generated using the neighbor-joining method (Saitou and Nei, 1987) and the MacVector and ClustalX (Thompson et al., 1997) programs. A random generator seed of 111 was used and 1000 bootstrap trials were generated. Trees were drawn using the MacVector and njplot programs.

2.5. Cisplatin viability curves

Cell viability was studied using the tetrazolium compound MTS (Promega) method. Briefly cells were seeded at 40,000 cells/well in a 24-well plate, 20–30 min later 0–200 μg/ml of cisplatin (CDDP) were added to the cultures. After 72 h, MTS was added to the cultures and the amount of degradation product determined by measuring optical absorption to 490 nm after three hours of incubation.

2.6. Folic acid induction and Western blot analysis

Folic acid induction of Erk2 phosphorylation was determined as previously described (Nguyen and Hadwiger, 2009). Cells were suspended in phosphate buffer (12 mM NaH2PO4, pH 6.1) at a concentration of 2×10^6 cells/ml, shaken for 1 h and stimulated with 50 μM folic acid. Cells were collected by centrifugation at the indicated times and suspended in SDS-PAGE sample buffer. Twenty μl of each extract were analyzed in 10% polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon P membranes (Millipore) and incubated with anti-phospho ERK antibodies (Thr202/Tyr204; Cell Signaling Technology). After ECL (Amersham) developing, blots were washed and incubated with anti-actin antibodies (Sigma, MI, USA) to normalize protein load. The phospho-ERK signal obtained for each sample was quantified by densitometry using the ImageJ program and divided by the signal obtained for the anti-actin antibody.

2.7. In vitro phosphatase activity assays

Phosphatase activity was determined using 3-O-methylfluorescein phosphate (OMFP) as a substrate. 2×10^7 cells were harvested and lysed in 1 mL NP-40 lysis buffer as described by Rice et al. (1997). The assay was performed in the buffer previously described, containing 45 μM OMFP, using 20 μg of total AX4, MKPB OE or MKPA OE protein, or 0.35 μg of purified human MKP1 protein (positive control) in the absence or presence of orthovanadate. Fluorescence emission was measured every 10 min of incubation at 30 °C using a multiwell plate reader (excitation/emission: 485 nm/530 nm). Fluorescence values were processed using SoftMax Pro v5 software. The reaction was linear over 2 h of incubation (V_{max}=0.071).

3. Results

3.1. Analysis of D. discoideum dual-specificity protein phosphatase coding genes

The phosphatase domain of the human dual-specificity protein phosphatase MKP1 (DUSP1) was used for a search of the proteins encoded in the *D. discoideum* genome. Fourteen proteins were found that presented significant similarity, with *E* values smaller than $6e^{-07}$ (Fig. 1). Some of these proteins had been described previously, like MkpA (Sawai et al., 2007) or MPL1 (Rodriguez et al., 2008). *D. discoideum* proteins were between 30% and 40% identical to the human MKP1 phosphatase domain. The percentage increased to 50–60% if conservative changes were admitted. Thirteen of the fourteen proteins contained the highly conserved catalytic domain HCXXXXXR. Only the protein encoded by the gene DDB0231326 presented changes in this region (CSDKGVSR) and might encode an inactive protein phosphatase.

The amino acid sequences of the putative phosphatase domains of these proteins were compared to those of the human dual-specificity protein phosphatases MKP1, MKP4 and slingshot3 domains and the multiple alignments used to construct the phylogenetic tree shown in Fig. 1a. The two *D. discoideum* proteins more similar to human MKP1 and MKP4 were encoded by DDB0202886 and DDB0238327/28(*mkpB*) genes. The two last genes (DDB0238327/28) are located in a chromosome-2 region that is duplicated in the AX4 strain used to determine the nucleotide sequence and code for identical proteins. The analysis of the domains present in the protein encoded by these two genes indicated the presence of a N-terminal Rhodanase domain that is characteristic of the mammalian MKP subfamily of DUSP phosphatases (Keyse and Ginsburg, 1993). This domain was only present in this protein among the 14 *D. discoideum* DUSP proteins. A search of the *D. discoideum* genome encoded proteins showed that only the cdc25 homologous protein contained this domain, in addition to the DDB0238327/28(*mkpB*) encoded protein. Fig. 1b shows the location of the two regions of the Rhodanase domain, as well as the phoshatase domain, on the DDB0238327/28(*mkpB*) encoded protein. The alignment of the two regions of the Rhodanase domain to those of human MKP1 and MKP4 and of the *D. discoideum* cdc25 proteins is shown in Fig. 1C. The presence of this N-terminal domain, and the similarity of the phosphatase domain, indicate that the protein encoded by the DDB0238327/28 genes is the *D. discoideum* protein more similar to mammalian MKP protein phosphatases so that the gene will be named *mkpB* and the protein MkpB in this article.

3.2. Determination of MkpB protein phosphatase activity

Functional characterization of MkpB was approached in AX4 cell populations over-expressing MkpB under control of the constitutive Actin 15 promoter. The same vector was used to over-express the previously characterized *mkpA* gene, used as a positive control. Cell populations derived from a large number of independent transformants were collected and used to determine phosphatase activity in vitro using the 3-O-methylfluorescein phosphate (OMFP) substrate and the results are shown in Fig. 2a. Two independent populations of cells over-expressing MkpB (MkpB OE1 and MkpB OE2) showed significantly more phosphatase activity than non-transformed AX4 cells. Cells over-expressing MkpA also showed more phosphatase activity.

The in vivo effects of MkpB over-expression on Erk2 phosphorylation was studied activating the cells with folic acid, that had been previously shown to induce Erk2 phosphorylation (Nguyen and Hadwiger, 2009). Concordantly, folic acid quickly induced Erk2 phosphorylation in AX4 cells reaching maximal levels by one minute to decrease thereafter (Fig. 2b). The cells that over-express MkpB showed lower Erk2 phosphorylation levels (Fig. 2b). These results indicate that MkpB over-expression result in a decrease in phosphorylated Erk2, in agreement with its expected function as a protein phosphatase.

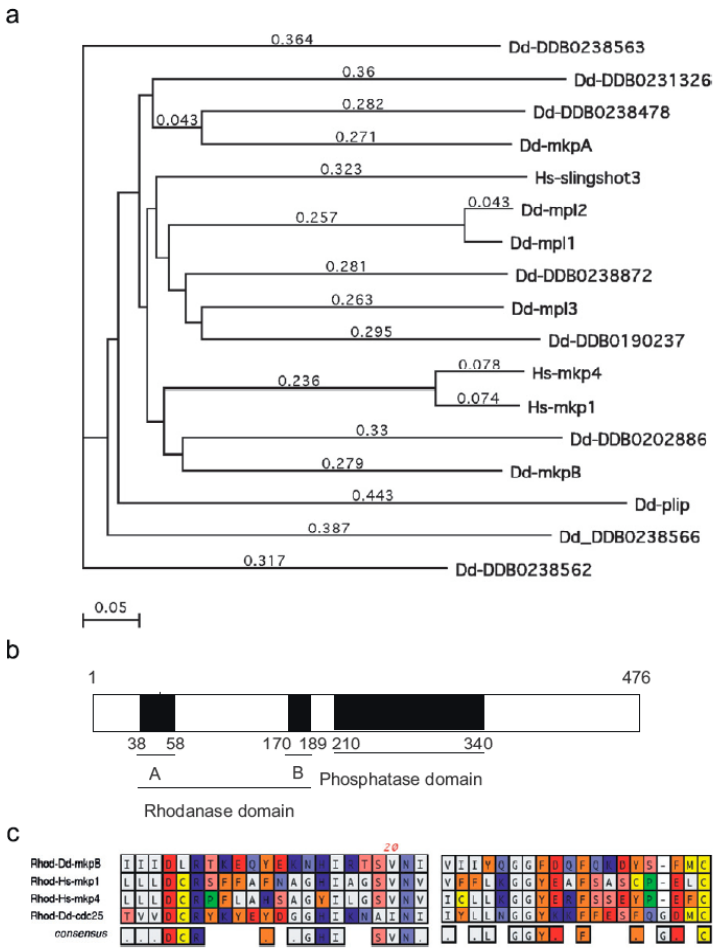

Fig. 1. *D. discoideum* genes coding for dual-specificity protein phosphatase homologous proteins. A search of the *D. discoideum* genome using the protein phosphatase domain of human MKP1 dual-specificity protein phosphatase detected the existence of 14 genes coding proteins with significant similarity. Panel A shows a phylogenetic tree indicative of the functional domain similarities among the *D. discoideum* proteins and with human MKP1, MKP4 and slingshot 3 dual-specificity protein phosphatases. Evolutionary distances, calculated as the fraction of nucleotide changes, are indicated over each branch and the scale is shown in the lower left corner. Bootstrap values over 850 were obtained for the Hs-mkp1/mkp4, DdmkpA/DDB0238478 and Ddmpl1/mpl2 associations. Panels B and C show the results obtained in a more detailed study of the *D. discoideum* gene DDB0238328, named *mkpB* in this article. Panel B shows the domain structure of the encoded protein, including the two regions of the Rhodanase domain (A, B) and the phosphatase domain. Domain limits are indicated underneath the scheme. Panel C indicates the alignment of the amino acid sequences of the A (left panel) and B (right panel) Rhodanase subdomains of *D. discoideum* MpkB and cdc25 and human Mkp1 and Mkp4 proteins.

3.3. Developmental expression of the mkpB gene

Expression of the *mkpB* gene in cells at growth and during multicellular development was analyzed by Northern blot (Fig. 3a). Low levels of expression were detected in growing cells and during the first hours of development but expression was strongly induced by 12 h of development and maintained at later developmental stages.

The possible cell-type specificity of *mkpB* expression was analyzed by cloning the complete intergenic region upstream of *mkpB* (1298 nt) in a *lacZ* reporter vector. *D. discoideum* AX4 cells were transfected with this reporter vector and *lacZ* expression analyzed at several developmental stages, as shown in Fig. 3b. *mkpB* promoter activity was detected in aggregates, being more intense in the upper, central part of the structures (Fig. 3b1). Later on during development promoter activity was detected in the whole structures although it was more intense in cells with a distribution typical of anterior-like cells. These cells were scattered in tight mounds, although they were more abundant in the basal region (Fig. 3b2). In finger and slug structures the more intensely stained cells were detected at the tip and scattered in the posterior prespore region (Fig. 3b3 and b4). In culminant structures the more intensive staining was observed in the basal disk, stalk, upper and lower cup regions (Fig. 3b5), as also described for anterior-like cells.

3.4. Analysis of mkpB function during development

The generation of mutant strains by homologous recombination was attempted to study MkpB function. However, this approach was not possible in AX4 cells, where this gene is

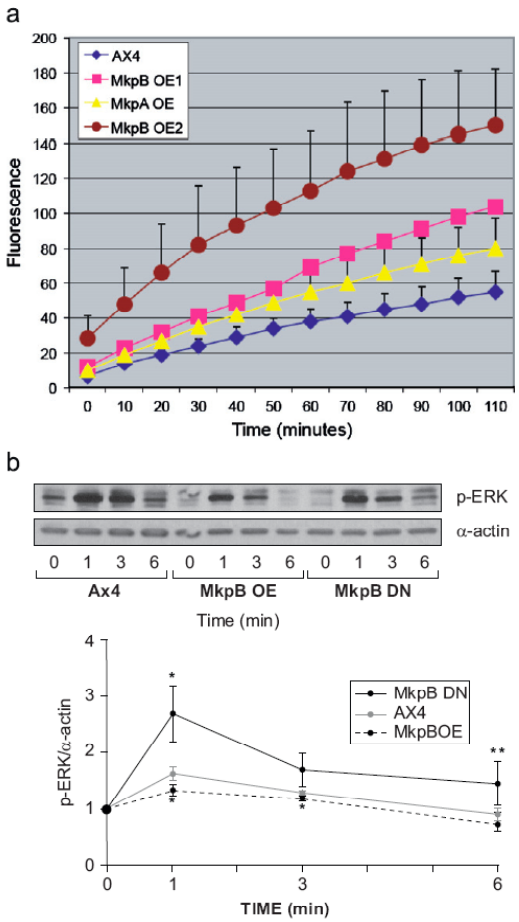

Fig. 2. Determination of MkpB phosphatase activity. Panel A: the phosphatase activity of extracts from AX4 cells or AX4 cells expressing MkpB (MkpB OE1, MkpB OE2) or MkpA (MkpA OE) was determined using 3-O-methylfluorescein phosphate (OMFP) as a substrate. Panel B: AX4 cells, either non-transfected or expressing MkpB (MkpB OE) or a catalytically inactive form of the enzyme (MkpB DN) were stimulated with folic acid and collected 1, 3 or 6 min thereafter. The presence of phosphorylated ErkB was analyzed by Western blots using anti-phospho Erk antibodies, as shown in the upper panel. The amount of phospho-ErkB was quantified by densitometry and normalized to the amount of actin present in each sample, as determined by Western blot. The results of the quantification are shown in the lower part of the panel. All data are representative of three independent experiments performed by triplicate.

duplicated, and was unsuccessful in AX2 cells, that contain a single copy of the gene. This circumstance forced the use of alternative approaches. Dual-specificity protein phoshatases require the presence of a conserved Cystein residue in their active center (Denu and Dixon, 1995; Zhou et al., 1994). In mammals, mutation of this residue originates catalytically inactive mutants that work as dominant-negative forms of the protein (Sanchez-Perez et al., 2000). Therefore, the codon corresponding to MkpB Cystein 294 (TGC) was changed to the Valine codon GCT. The inactive form of the protein (MkpB-DN) was expressed in AX4 cells under the control of the constitutive Actin 15 promoter. The possible consequences of the expression of this catayticaly inactive form on Erk activity was analyzed in vivo in response to folic acid. The cells that expressed the catalytically inactive form showed a larger increase in Erk phosphorylation in response to folic acid than AX4 cells (Fig. 2b) in agreement with the proposed inactivating effect of the expression of this mutated MkpB form. Therefore, these cells have been named MkpB-DN (dominant negative) in the rest of the article.

Cells expressing MkpB-DN did not show any differences with the original cells in their growth, either in axenic media or feeding on bacteria (data not shown). Multicellular development was studied laying starved cells on plastic dishes (Fig. 4a) or over Nitrocellulose filters (Fig. 4b). Two different pools of cells expressing MkpB-DN, derived from independent transformations, were used in these studies (MkpB-DN1 and MkpB-DN2). These cells polarized during aggregation on plastic dishes and associated into streams (Fig. 4a) although they were less tightly packed than non-transformed cells (compare lower panels in Fig. 4a). In agreement with these data, MkpB-DN cells aggregated by 12 h of development on Nitrocellulose filters (Fig. 4b). However, post-aggregative development was greatly impaired in MkpB-DN expressing cells. Most of the aggregates did not continue development and those that progressed further formed very small slug (Fig. 4b, 20 h) and small culminant structures with thickened stalks, even after 48 h of development (Fig. 4b, 24 and 48 h). This phenotype was not due phosphatase activity-independent effects of MkpB over-expression because expression of non-mutated MkpB using the same vector did not produce any alteration in development (Fig. 4b).

Postaggregation development is a coordinated process that involves differentiation of prespore and prestalk cells, morphogenetic movements and, later on, the terminal differentiation of stalk cells and spores. To determine which of these processed was altered by MkpB-DN the expression of several developmental markers was studied. The expression of gene coding for the cAMP receptor 1 (*carA*) is induced at aggregation in AX4 cells to decrease at later developmental stages (Fig. 5a, AX4). The cells that express MkpB-DN showed *carA* induction at aggregation with a small delay of two to four hours in relation to AX4 cells (Fig. 5a, MkpB-DN1 and MkpB-DN2). *carA* mRNA levels also decreased from 16 h of development in MkpB-DN expressing cells although significant expression was maintained in MkpB-DN1 cells up to 24 h of development. The prestalk gene *ecmB* (coding for an extracellular matrix protein) and the prespore gene *cotD* (coding for a protein of the spore coat) are induced in AX4 cells after aggregation (10–12 h of development) while mRNA levels decrease at culmination (24 h), as shown in Fig. 5b and c. The cells that express MkpB-DN showed an important delay in the expression of both markers (Fig. 5b and c). These data indicate that MkpB-DN expression originated a marked delay in the differentiation of both prespore and prestalk cells that might explain the blockage observed in post-aggregative development.

3.5. *MkpB involvement in D. discoideum response to cisplatin*

Cisplatin (CDDP) is one of the antitumoral drugs more relevant in clinical treatments and the human MKP1 protein is involved in the mechanism of resistance to this compound. For example, MKP1 is expressed in non-small-cell lung cancer and decreasing its expression significantly increases the sensitivity of these cells to CDDP (Chattopadhyay et al., 2006; Cortes-Sempere et al., 2009). To know if *D. discoideum* MKPs could play a similar role both MkpA and MkpB proteins were overexpressed in AX4 cells. Cells over-expressing *D. discoideum* MkpA or MkpB showed increased resistance to CDDP, in comparison to AX4 cells (Fig. 6a). On the contrary, expression of MkpB-DN protein produced

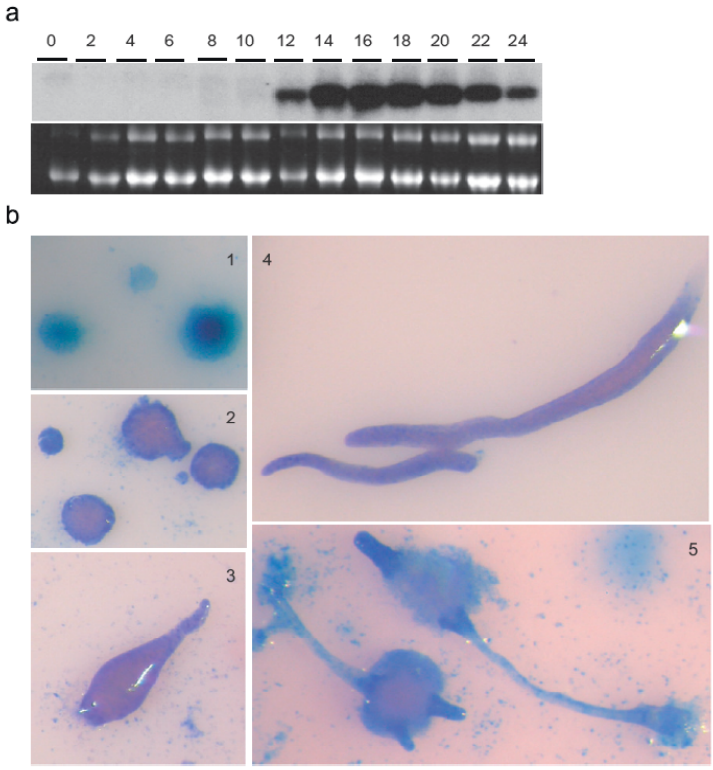

Fig. 3. Expression of the *mkpB* gene. Panel A: temporal *mkpB* mRNA expression in growing cells (0) and at different times of multicellular development (2–24 h) was analyzed by Northern blot. The upper panel shows the hybridization signals and the lower panel the Ethidium Bromide staining of the RNA gel. Panel B: the promoter region of the *mkpB* gene was cloned in a *lacZ* reporter vector. Developmental structures obtained from cells transformed with this vector were stained to detect *lacZ* activity and counterstained with eosine. The structures shown correspond to the early aggregate (panel B1), tight aggregate (B2), finger (B3), slug (B4) and culminant (B5) stages of development.

increased sensitivity to CDDP, as shown in Fig. 6b. These data indicated that MAP kinase activity is involved in CDDP sensitivity. To further test this possibility, cells mutated in one of the two genes coding for *D. discoideum* MAP kinases, erkB, were analyzed and showed to be significantly more resistant to CDDP than wild type AX4 cells (Fig. 6b). These results indicated that the mechanisms of response to CDDP involving MAP kinases and MKP proteins are conserved between humans and *D. discoideum* and that this ameba could be a good model system to study the implication of these proteins in CDDP resistance.

4. Discussion

The gene *mkpB* codes for the *D. discoideum* protein more similar to animal MAP kinase phosphatases (MKPs), among the 14 genes that code for dual-specificity protein phosphatase domain containing proteins in this organism. This conclusion is based on the similarity of the MkpB protein phosphatase domain with those of mammalian MKP1 and MKP4 proteins that makes them group together in the phylogenetic tree shown in Fig. 1, in addition to the DDB020286-encoded protein. The second argument is the presence of a region with similarity to the Rhodanase domain, characteristic of animal MKPs (Keyse and Ginsburg, 1993), in the N-terminal region of MkpB. This domain was only found in MkpB among the 14 *D. discoideum* dual-specificity protein phosphatases. In addition, only MkpB and Cdc25 presented a Rhodanase domain among *D. discoideum* proteins. Cells over-expressing MkpB showed increased phosphatase activity in in vitro assays. Besides, Erk2 phosphorylation in response to folic acid stimulation was reduced in MkpB-over-expressing cells, indicating that the *mkpB* gene codes for a functional MAP kinase phosphatase protein.

This gene is expressed in replicating ameba and at all stages of the developmental process induced by starvation. However, its expression is strongly induced at 12 h of development, after the aggregation process is completed. Analysis of reporter vectors containing the promoter region indicated that *mkpB* is expressed at all the regions of the structure although the highest levels of expression are found in cells with the same distribution as anterior-like cells. This cell population show a scattered distribution in slug and finger structures and form part of the basal disk, stalk, lower and upper cup and tip regions in culminant structures.

The study of *mkpB* function was initially approached through the generation of mutant strains by homologous recombination. However, this approach was not possible in AX4 cells, because the gene is duplicated in this strain, and was unsuccessful in AX2 cells. The alternative strategy of expressing an inactive form of the protein (MkpB-DN) previously described for mammalian

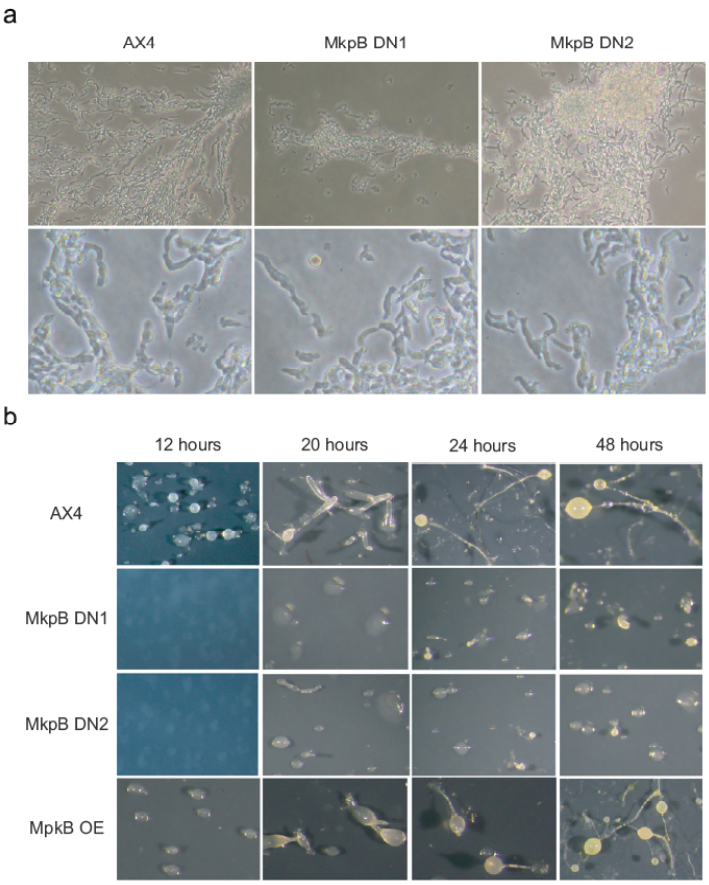

Fig. 4. Multicellular development of cells expressing a catalytically inactive form of MkpB. *D. discoideum* AX4 cells were transformed with a vector expressing MkpB (MkpB OE) or a catalytically inactive form of MkpB (MkpB-DN) under the control of the constitutive Actin 15 promoter. AX4 cells or two populations of transformed cells (MkpB DN1, MkpB DN2) were harvested and laid on plastic dishes (panel A) or Nitrocellulose filters (panel B) to observe multicellular development. AX4 cells transformed with MkpB (MkpB OE) were also laid on Nitrocellulose filters (panel B). The images of panel A were obtained at 12 h of development while those of panels B were taken at the times indicated in the upper part of the panels.

MKPs (Denu and Dixon, 1995) was, therefore, chosen. Cells expressing the MkpB inactive form of the protein grew similarly to AX4 cells both in axenic media and feeding on bacteria, which would indicate that MkpB is not required for cell proliferation. Multicellular development was, however, greatly impaired. Cells expressing MkpB-DN initiated aggregation at about the same time as AX4 cells, polarized and formed streams timely, although they were not as tightly packed in the streams as AX4 cells.

Most of the aggregates formed by MkpB-DN-expressing cells did not progress any further in the developmental process, even after 48 h from starvation. The few aggregates that proceeded further formed very defective structures. The morphological alterations were reflected in the pattern of expression of differentiation marker genes. Cells expressing MkpB-DN expressed the *carA* aggregation-specific gene with a temporal pattern similar to that of AX4 cells, except for a small delay of about two to four hours. However, one prestalk (*ecmB*) and one prespore (*cotD*) gene, whose expression is induced after aggregation, showed a marked delay with respect to AX4 cells. Besides, the expression of these genes remained at high levels at 24 h of development while their expression decreased in AX4 cells after culmination was completed. This result is in agreement with the morphological observation that MkpB-DN expressing cells remained at the mound stage of development. These data also indicate that the developmental delay observed was not limited to specific defects in prestalk or prespore cell differentiation but to a very general blockage of differentiation and morphogenesis. However, the phenotype could be initially due to impairment of prestalk or prespore cell differentiation since a block in one cell type often interferes with the development of another cell type.

Dual-specificity protein phosphatases regulate the activity of MAP kinases, inactivating them. The defects observed in MkpB-DN expressing cells are expected to be due to interference with the MAP kinase regulatory pathways that regulate development. The existence of 14 *D. discoideum* genes coding for MAP kinase phosphatase homologous proteins makes possible that the phenotype observed could be due to interference with other phosphatases, in addition to or instead MkpB. However, the temporal coincidence of *mkpB* expression and the defects observed is consistent with the phenotype being due to interference with MkpB-dependent regulatory pathways.

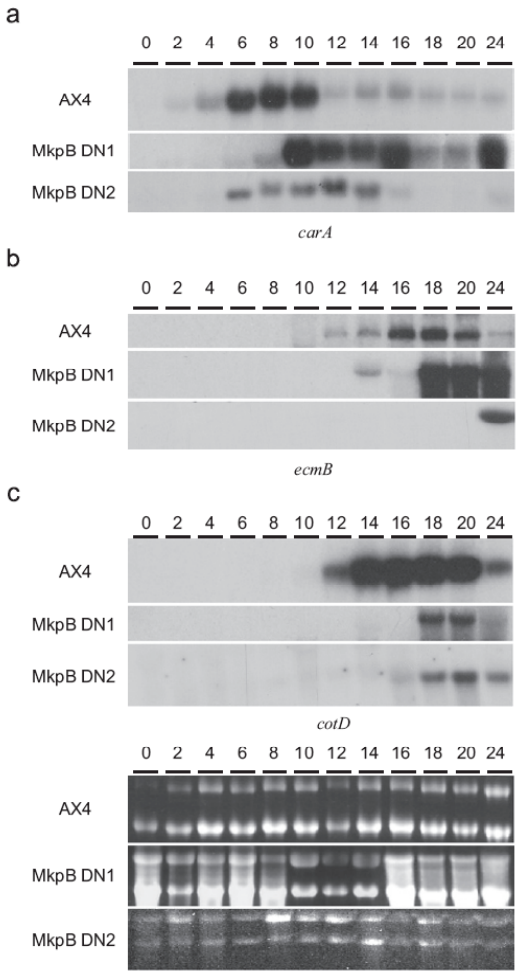

Fig. 5. Analysis of gene expression during development of cells expressing a MkpB catalytically inactive form. RNA was extracted from AX4 cells or two populations of AX4 cells expressing a MkpB catalytically inactive form (MkpB DN1, MkpB DN2) during growth (0) or after 2–24 h of development (2–24). The expression of the aggregation-specific gene *carA*, coding cAMP receptor 1 (panel A), the prestalk *ecmB* gene, coding for the extracellular matrix protein EcmB (panel B) or the prespore-specific *cotD* gene, coding for the spore coat protein CotD (panel C) was analyzed by Northern blot. The lower panel shows the Ethidium Bromide staining of the gels.

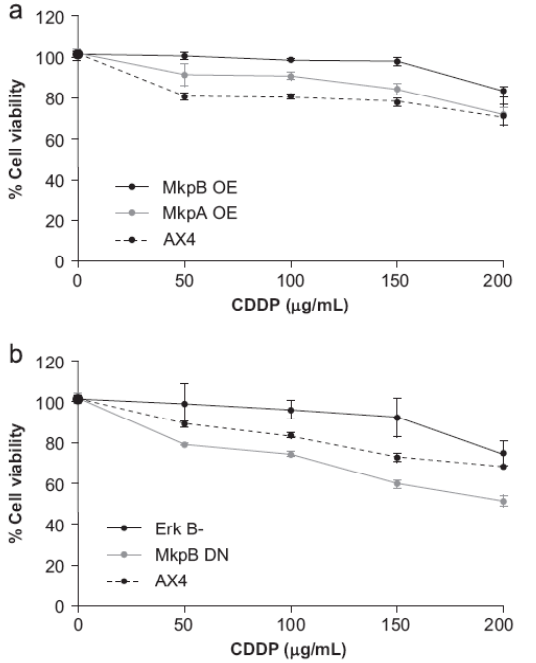

Fig. 6. Cisplatin sensitivity of *D. discoideum* cells expressing wild type or catalytically inactive Mkp proteins. Panel A: AX4 cells non-transfected (AX4), or transformed with vectors where the expression of MkpA (MkpA OE) or MkpB (MkpB OE) was regulated by the constitutive Actin 15 promoter. Cells were incubated in the presence of 0–200 μg/ml of cisplatin (CDDP) for 48 h. The number of viable cells was estimated by MTS hydrolysis after 48 h of treatment and represented as the percentage of untreated cells. Panel B: AX4 cells (AX4), a population of AX4 cells expressing a catalytically inactive form of MkpB (MkpB DN) or erkB-mutant cells (Erk B-) were incubated in the presence of 0–200 μg/ml of cisplatin (CDDP) for 48 h and the MTS assay performed. The percentage of viable cells was calculated as indicated in panel A. All data are representative of three independent experiments performed by triplicate.

As mentioned in the Introduction section, only two genes coding for MAP kinases have been identified in *D. discoideum*, *erkA* and *erkB*, and both genes are required for regulation of development. *ErkA* is expressed at growth but its expression is also induced from 8 h of development under the control of a development-specific promoter (Gaskins et al., 1994). This promoter drives *erkA* expression in cells with a distribution similar to that of anterior-like cells and also very similar to the one observed for cells with maximal *mkpB* expression, as mentioned above. Cells where the *erkA* genes has been mutated form small aggregates that, in the most part, do not continue development (Nguyen et al., 2010; Sobko et al., 2002), as also observed for cells expressing MkpB-DN.

Cells mutated in the *erkB* gene do not aggregate because Erk2 is required for establishment of the cAMP oscillatory waves that regulate this process (Maeda et al., 2004). In addition, Erk2 is also required for prespore cells differentiation at the mound stage of development, in a process that is independent of cAMP signaling (Gaskins et al., 1996).

Two dual-specificity protein phosphatases with the capacity to regulate Erk1 and Erk2 activity have been characterized in *D. discoideum*, MPL1 and MkpA. *Mpl1* expression is induced by 5 h of development and mutant cells show defective streaming and aggregation (Rodriguez et al., 2008). Similarly, *mkpA* mutant cells show impaired aggregation (Sawai et al., 2007), suggesting that these two protein phosphatases regulate the aggregation process that is dependent on Erk2 activity. On the contrary, *mkpB* expression is induced after aggregation and expression of MkpB-DN does not significantly alter the aggregation process, indicating that this protein phosphatase rather regulates cell differentiation and morphogenesis. We would like to suggest that MkpB might be responsible for Erk1 inactivation, given that both proteins show similar expression patterns and Erk1 has been related to cell differentiation and tip formation, as mentioned above. Expression of the catalytically inactive mutant is expected to result in sustained Erk1 activation and the functional consequences could be the opposite of *erkA* mutation. However, long-lasting

stimulation of MAP kinases can also negatively affect the involved signaling pathways and the consequences on Erk1-overstimulation have not been described. In addition, MkpB could also regulate Erk2 activity during spore differentiation since MkpB-DN expressing cells show a strong delay in the expression of the *cotD* prespore gene. Obviously, further experiments will be necessary to confirm or reject these hypotheses.

The last part of the article approaches the study of a different function that has been described for mammalian MKP proteins. A strong correlation between MKP1 activity and cisplatin resistance has been shown in human cancer cells. Cells with decreased Mkp1 expression or over-expressing a dominant-negative form of the enzyme are more sensitive to cisplatin while cells that over-express this protein are more resistant to the drug (Chattopadhyay et al., 2006; Sanchez-Perez et al., 2000). The same effect was observed for two *D. discoideum* MAP kinase phosphatases, MkpA and MkpB. Cells over-expressing any of these phosphatases are more resistant to cisplatin. On the contrary, cells that express a MkpB catalytically inactive form are more sensitive. Furthermore, cells mutated in the MAP kinase-coding gene erkB are more resistant to cisplatin than wild type cells. In agreement with these data, ErkB regulates the activity of the cAMP phosphodiesterase RegA (Maeda et al., 2004), that has also been involved in cisplatin resistance in *D. discoideum* (Li et al., 2000; Niedner et al., 2001). These observations indicate that MAP kinase- and MKP-dependent cisplatin resistance mechanisms have been conserved through evolution and makes of *D. discoideum* a good model system for their study.

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References

Annesley, S.J., Fisher, P.R., 2009. *Dictyostelium discoideum*—a model for many reasons. Mol. Cell Biochem. 329, 73–91.

Chattopadhyay, S., Machado-Pinilla, R., Manguan-Garcia, C., Belda-Iniesta, C., Moratilla, C., Cejas, P., Fresno-Vara, J.A., de Castro-Carpeno, J., Casado, E., Nistal, M., Gonzalez-Baron, M., Perona, R., 2006. MKP1/CL100 controls tumor growth and sensitivity to cisplatin in non-small-cell lung cancer. Oncogene 25, 3335–3345.

Cortes-Sempere, M., Chattopadhyay, S., Rovira, A., Rodriguez-Fanjul, V., Belda-Iniesta, C., Tapia, M., Cejas, P., Machado-Pinilla, R., Manguan-Garcia, C., Sanchez-Perez, I., Nistal, M., Moratilla, C., Castro-Carpeno, J.D., Gonzalez-Baron, M., Albanell, J., Perona, R., 2009. MKP1 repression is required for the chemosensitizing effects of NF-kappaB and PI3K inhibitors to cisplatin in non-small cell lung cancer. Cancer Lett. 286, 206–216.

Denu, J.M., Dixon, J.E., 1995. A catalytic mechanism for the dual-specific phosphatases. Proc. Natl. Acad. Sci. USA 92, 5910–5914.

Dickinson, R.J., Keyse, S.M., 2006. Diverse physiological functions for dual-specificity MAP kinase phosphatases. J. Cell Sci. 119, 4607–4615.

Eichinger, L., Pachebat, J.A., Glockner, G., Rajandream, M., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., 2005. The genome of the social amoeba *Dictyostelium discoideum*. Nature 435, 43–57.

Escalante, R., Sastre, L., 2006. Investigating gene expression: in situ hybridization and reporter genes. In: Eichinger, L., Rivero, F. (Eds.). *Dictyostelium discoideum* Protocols. Humana Press, Totowa, NJ, pp. 230–247.

Gaskins, C., Maeda, M., Firtel, R.A., 1994. Identification and functional analysis of a developmentally regulated extracellular signal-regulated kinase gene in *Dictyostelium discoideum*. Mol. Cell. Biol. 14, 6996–7012.

Gaskins, C., Clark, A.M., Aubry, L., Segall, J.E., Firtel, R.A., 1996. The Dictyostelium MAP kinase ERK2 regulates multiple, independent developmental pathways. Genes Dev. 10, 118–128.

Harwood, A.J., Drury, L., 1990. New vectors for expression of the *E. coli* lacZ gene in Dictyostelium. Nucl. Acids Res. 18, 4292.

Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911–1912.

Keyse, S.M., 2008. Dual-specificity MAP kinase phosphatases (MKPs) and cancer. Cancer Metastasis Rev. 27, 253–261.

Keyse, S.M., Ginsburg, M., 1993. Amino acid sequence similarity between CL100, a dual-specificity MAP kinase phosphatase and cdc25. Trends Biochem. Sci. 18, 377–378.

Li, G., Alexander, H., Schneider, N., Alexander, S., 2000. Molecular basis for resistance to the anticancer drug cisplatin in Dictyostelium. Microbiol. UK 146, 2219–2227.

Maeda, M., Lu, J., Shaulsky, G., Miyazaki, Y., Kuwayama, H., Tanaka, Y., Kuspa, A., Loomis, W., 2004. Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA. Science 304, 875–878.

Marshall, C.J., 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

Meima, M.E., Weening, K.E., Schaap, P., 2007. Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium. Protein Expression Purif. 53, 283–288.

Nguyen, H.N., Hadwiger, J.A., 2009. The Galpha4 G protein subunit interacts with the MAP kinase ERK2 using a D-motif that regulates developmental morphogenesis in Dictyostelium. Dev. Biol. 335, 385–395.

Nguyen, H.N., Raisley, B., Hadwiger, J.A., 2010. MAP kinases have different functions in Dictyostelium G protein-mediated signaling. Cell Signal. 22, 836–847.

Niedner, H., Christen, R., Lin, X., Kondo, A., Howell, S.B., 2001. Identification of genes that mediate sensitivity to cisplatin. Mol. Pharmacol. 60, 1153–1160.

Pang, K.M., Lynes, M.A., Knecht, D.A., 1999. Variables controlling the expression level of exogenous genes in Dictyostelium. Plasmid 41, 187–197.

Patterson, K.I., Brummer, T., O'Brien, P.M., Daly, R.J., 2009. Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem. J. 418, 475–489.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22, 153–183.

Qi, M., Elion, E.A., 2005. MAP kinase pathways. J. Cell Sci. 118, 3569–3572.

Rice, R.L., Rusnak, J.M., Yokokawa, F., Yokokawa, S., Messner, D.J., Boynton, A.L., Wipf, P., Lazo, J.S., 1997. A targeted library of small-molecule, tyrosine, and dual-specificity phosphatase inhibitors derived from a rational core design and random side chain variation. Biochem. 36, 15965–15974.

Rodriguez, M., Kim, B., Lee, N.S., Veeranki, S., Kim, L., 2008. MPL1, a novel phosphatase with leucine-rich repeats, is essential for proper ERK2 phosphorylation and cell motility. Eukaryot. Cell 7, 958–966.

Saitou, N., Nei, M., 1987. The neighbor-joining method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sambrook, J., Frisch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sanchez-Perez, I., Martinez-Gomariz, M., Williams, D., Keyse, S.M., Perona, R., 2000. CL100/MKP-1 modulates JNK activation and apoptosis in response to cisplatin. Oncogene 19, 5142–5152.

Sawai, S., Guan, X.J., Kuspa, A., Cox, E.C., 2007. High-throughput analysis of spatio-temporal dynamics in Dictyostelium. Genome Biol. 8, R144.

Segall, J.E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R.A., 1995. A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in Dictyostelium. J. Cell Biol. 128, 405–413.

Sobko, A., Ma, H., Firtel, R.A., 2002. Regulated SUMOylation and ubiquitination of DdMEK1 is required for proper chemotaxis. Dev. Cell 2, 745–756.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougins, F., Higgins, D., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.

van Haastert, P.J.M., Devreotes, P.N., 2004. Chemotaxis: signalling the way forward. Nat. Rev. Mol. Cell Biol. 5, 626–634.

Zheng, L., Baumann, U., Reymond, J.L., 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucl. Acids Res. 32, e115.

Zhou, G., Denu, J.M., Wu, L., Dixon, J.E., 1994. The catalytic role of Cys124 in the dual specificity phosphatase VHR. J. Biol. Chem. 269, 28084–28090.